12-2009

Investigating Nectar Rhythms in Squash (*Cucurbita pepo*): Effects on Honey Bee (*Apis mellifera*) Foraging Behavior.

Samuel David Boyd
East Tennessee State University

Investigating Nectar Rhythms in Squash (*Cucurbita pepo)*: Effects on Honey Bee (*Apis mellifera*) Foraging Behavior

---

A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Masters of Science in Biology

---

by

Samuel D. Boyd

December 2009

Dr. Darrell Moore, Chair

Dr. Thomas Jones

Dr. Thomas Laughlin

Keywords: nectar, *Cucurbita pepo*, *Apis mellifera*, foraging behavior, time-memory

ABSTRACT

Investigating Nectar Rhythms in Squash (*Cucurbita pepo)*: Effects on Honey bee (*Apis mellifera*) Foraging Behavior

by

Samuel D. Boyd

Experiments were performed to investigate the influence of water availability on the diel patterns of nectar secretion (volume, concentration, sugar production) in male squash flowers as well as to discover what physical component of nectar honey bees use to trigger their time-memory. Squash plants were grown in the greenhouse and in the field under both constant and variable watering regimes. Throughout anthesis, nectar volume and sugar concentration were recorded. In the field, the temporal distribution of arrivals to squash was observed with and without blossoms present. In the greenhouse and in the field, squash flowers exhibit a consistent diel pattern of nectar secretion that does not significantly alter during drought conditions; flowers open just before sunrise (with low volume and sugar and high concentration) and close at midday (with high volume and sugar and low concentration). Honey bees preferentially arrived early in anthesis possibly cueing on either the sugar concentration or the first availability of nectar.

ACKNOWLEDGEMENTS

First of all I would like to first thank all of the people who endured much through heat and cold to conduct my various experiments. Without their tireless work and encouragement this would not have been possible. These wonderful people include my lab mates: Byron N. van Nest, Jennifer N. Johnson, Andrea A. Edge, and Caleb Paquette as well as help from Huijing Sun, Joanna Magner, and Curtis Gill.

Second, I would like to thank my committee for all of their helpful input in the design stage of this work. Specifically, I would like to thank my chair, Dr. Moore, for his continued support and writing expertise in helping in the editing process of this manuscript.

For funding this work I would like to acknowledge the Department of Biological Science for allowing me conduct my research with its prolonged financial support. I would also like to thank the USDA for its support as well.

Last, my thanks go out to my friends and family for all their encouragement and support without which none of this would have been possible.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The vast majority of the honey bee (*Apis mellifera*) hive is made up of the worker caste. These females usually do not reproduce but perform most of the duties in the colony. Normally, workers progress through tasks based on their age, although this behavioral ontogeny is modulated by the needs of the colony (Schultz et al. 1998). This age related division of labor, called age polytheism, shows a progression of young bees from in-hive tasks such as cell cleaning and nectar receiving, tasks that are more dangerous and require more time spent outside the hive such as guarding the hive entrance and foraging (Seeley 1995). Foragers are tasked with the role of collecting all the external products required for the survival of the hive including pollen and nectar, the protein and carbohydrate sources required for the proper maintenance and growth of the colony (Winston 1987).

As "sensory units" for the hive, foragers provide necessary resources as well as vital information about the foraging environment (Seeley 1994). Each individual forager falls into one of two categories, employed or unemployed, based on whether or not the forager is currently exploiting a floral source. Using recruitment dances, employed foragers provide information about the location and profitability of the floral source that they are currently exploiting (von Frisch 1967).

Each individual nectar forager is able to assess the profitability of her food source by using a number of criteria including the concentration of sucrose and the distance from the hive (Seeley et al. 1991). Successful foragers, upon returning to the hive, will attempt to unload their payload to food processing bees. It has been shown that food processing bees will unload nectar

foragers in order of highest to lowest sugar concentration (Seeley et al. 1991). This feedback loop allows each forager to assess the specific relative profitability of her patch in relation to other patches currently being exploited by the hive. Based on this information, each employed forager decides whether to advertise her source to unemployed foragers in the hive.

Von Frisch (1967) determined that foragers are sensitive to changes in the nectar concentration. They operate with a dance threshold, nectar sources with sugar concentrations below this threshold will not be advertised. This threshold is not static but shifts based on the foraging environment. This allows the hive to properly adjust to changes in the foraging environment. Operating at a lower dance threshold gives the hive input from a wider variety of sources. On the other hand, abundant forage will cause the threshold to rise, filtering out inputs from low quality forage sites (Seeley 1994). Because only profitable sources are being advertised, the colony is able to preferentially allocate its foragers to the most rewarding sources.

Because the sugar concentration of a floral source fluctuates temporally (Wyatt et al. 1992), knowing the current profitability of each source is of utmost importance to the hive. Seeley et al. (1991) found that honey bees quickly adapt their foraging behavior when the profitability of an artificial source changes. As a source becomes less attractive such as with a decrease in sugar concentration, a greater number of bees will abandon the source. In addition, fewer foragers will be advertising for this source, thus lowering the number of new recruits. Similarly, as a source becomes more profitable, the number of bees exploiting the patch will increase as more foragers advertise this source.

A circadian rhythm is an endogenous physiological, biochemical, or behavioral process that occurs with a period of roughly 24 hrs. For a rhythm to genuinely be considered endogenous, the rhythm must persist or “free run” in the absence of external cues showing the

actual length of its period, which is usually around 24 hrs in length (Saunders 2002). The actual period of the free running rhythm for most organisms deviates slightly from 24 hr, which it has been asserted aides in its ability to be entrained by an external time cue (Moore-Ede et al. 1984). The presence of an endogenous clock allows organisms to perform tasks at "the right time of day" with adaptive consequences. Each day the time cue (such as feeding cycle, light dark cycle, etc.) will reset the circadian clock such that the organism's rhythm and the external time cue adapt the same period.

Honey bee workers begin life arrhythmic and develop a circadian rhythm prior to foraging. Young workers that perform in-hive tasks must complete these duties whenever needed. Queen care and brood care, for example, require around-the-clock attention (Moore et al. 1998). Therefore, the lack of an endogenous clock for young workers is advantageous to the overall health of the colony. Conversely, a circadian rhythm is highly adaptive for foragers, that must perform their tasks exclusively during daylight hours.

It has been known since the turn of the 20th century that honey bee foragers are able to associate the time of day with a food reward (Buttel-Reepen 1900; Forel 1910). This ability to associate the time of day with the presence and location of food is known as the time-memory. Although, the observation of this phenomenon was first accredited to Buttel-Reepen (1900) and Forel (1910), it was not until a series of classical experiments by Beling that this hypothesis was empirically tested. Beling (1929) found that honey bees could be trained to visit a feeding dish at a certain time of day. Furthermore, studies conducted under constant conditions (light, temperature, and humidity) showed that the foraging rhythm persisted, indicating the endogenous nature of the time-sense (Beling 1929; Wahl 1932).

The accuracy of the time-memory shows diurnal variation. Taken as a population

response, the time-memory is assayed by noting the temporal distribution of arrivals to an artificial feeder on a test day in which no reward is given. In time-memory studies forager arrivals increase in frequency as the onset of the training time approaches and rapidly decline afterward (Beling 1929; Wahl 1932; Moore and Rankin 1983). It has been demonstrated that the duration of this anticipatory behavior varies according to time of day (Moore and Rankin 1983). The length of anticipation is greatest in the afternoon and least in the morning hours. This shows that the time-sense is most accurate early in the day.

The time-memory is an essential component of honey bee foraging behavior. The malleability of the foragers' time-sense increases foraging efficiency. Foragers with as little as 1 day of training are able to learn up to nine different feeding times a day (Koltermann 1974). In addition, foragers are able to link different times of day to differences in the quality of food (different sugar concentrations) encountered at an artificial source (Wahl 1933). Instead of wasting energy on unproductive flights, foragers are able to schedule their foraging flights to coincide with species-specific daily periods of nectar secretion and pollen availability. In this way the time-sense is highly adaptive as it allows foragers to learn when a food source is most profitable and return during that time period the following day.

A single successful foraging trip may be adequate for acquisition of the time-memory (Moore and Doherty, in prep.); however, once the food reward is removed, this time-memory for individual foragers begins to diminish over several days. The more days of training the bees receive, the longer it takes for this apparent extinction to take place. Following a rain event, a larger than expected proportion of foragers returned to the feeding station showing that the extinction of the time-memory was delayed (Van Nest, Seier, and Moore, in prep.). It was observed that for bees with 2, 3, and 4 days of training a significantly greater proportion returned

after a 2-day rain event than would be expected under normal weather conditions. One of our goals with this present study is to investigate whether this delay in extinction may be linked to a rebound effect on nectar quality (an increase in nectar secretion or an increase in the sugar concentration of the nectar) due to a rain event.

Temporal fluctuations in nectar quality such as changes in nectar concentration or volume will affect the timing of foraging flights to floral sources. Anthesis is defined as the time period in which the flower is open and floral rewards are available. In Butternut squash (*Cucurbita moschata*), honey bee foraging activity does not extend through all of anthesis. Instead, visits are restricted to a window 2 to 3 hrs after the blossoms open, which correlates to the period of time when the rate of nectar secretion is the largest (Canto-Aguilar and Parra-Tabla 2000). In a similar finding, Nepi and Pacini (1993) found that honey bee visits to Zucchini (*Cucurbita pepo.* variety 'Greynzini') peak early in anthesis and decline rapidly, ceasing completely before the end of anthesis. Clearly, in order to fully understand factors contributing to the timing of forager visits, more must be known about the temporal patterns of nectar secretion in floral sources.

For most plant species a complete description of nectar secretion patterns is lacking. This is because a number of factors affect nectar production. Genetic variation as well as the sex of the flower may affect nectar production. For example, different breeding lines within the single species *Capsicum annuum* demonstrated a significant difference in the volume of nectar production (Rabinowitch et al. 1993). Nepi et al. (2001) determined that in *C. pepo,* female flowers produce a significantly larger volume of nectar than do their male counterparts. Water availability is another factor that affects nectar production in several species such as in *Asclepias* species. As precipitation increases volume of nectar also increases in *A. syriaca* and *A. exaltata* (Wyatt et al. 1992). Similarly, as water decreases nectar production decreases in *Epilobium*

angustifolium and *E. canum* (Boose 1997; Carroll et al. 2001). Time of day also affects production as daily rhythms of nectar secretion differ between plant species. For example, with pollinator activity present, *Cucurbita moschata* blossoms open with very little nectar, gradually producing larger volumes for about 3 hours, after which the volume declines until the blossoms close (Canto-Aguilar and Parra-Tabla 2000). In *C. pepo*, blossoms begin secreting nectar prior to blossom anthesis and continue secreting until they close (Nepi et al. 2001).

Despite the importance of pollination to plants and animals, there has been little research conducted on nectar rhythms in plants. Consequently, a complete understanding of the dynamics of nectar production and consumption is lacking for any known species of plant (Corbet and Delfosse 1984). While Canto-Aguilar and Parra-Tabla (2000) found that honey bee foraging visits correlate to the time of highest nectar secretion rate in *Cucurbita moschata*, they did not address changes in nectar concentration. Wyatt et al. (1992) used *Asclepias syriaca* and *A. exaltata* to describe diel trends of nectar volume and concentration; however, the relationship between these nectar qualities was not associated with pollinator visits. In a study looking at *Delphinium nelsonii*, Zimmerman (1983) demonstrates a positive correlation in the frequency and duration of forager visits to blossoms with larger standing crops. However, nectar samples were taken only once every 24 hrs and the temporal distribution of pollinator activity was not given.

The standing crop of a flower at any given time is a function of the rate of nectar production and the time elapsed since the flower was last visited by a pollinator. However, further complicating matters is nectar removal by pollinators, which has been shown to affect nectar secretion. For example, in *Penstemon speciosus* nectar removal stimulates replenishment (Castellanos et al. 2002), while for other plant species such as *Ipomopsis aggregate* no such

effect has been observed (Pleasants 1983). Therefore, the standing crop of nectar in the field is not only affected by the quantity of pollinator visits but also by the species of plant under investigation.

The wealth of knowledge accumulated on the time-memory of honey bees has led to many questions that have yet to be answered. The ability of foragers to learn and anticipate a food reward from an artificial feeder via the time-memory has been thoroughly documented. However, little is known about how the time-memory is formed to a natural floral source. Furthermore, it has yet to be determined how consistent nectar rhythms are from species to species or even from flower to flower of the same species. In addition, it is unknown how these species-specific nectar trends will react to changes in the environment. Consequently, these gaps in our knowledge lead us to question what physical component of nectar honey bees use to form their time-memory to a natural source that shows diurnal fluctuations in both nectar quantity and quality.

This present study seeks to: (1) investigate the diel secretion patterns of nectar volume, sugar concentration, and sugar production for *Cucurbita pepo* under varying environmental conditions; (2) determine the response of honey bees to squash nectar rhythms in the field, identifying what component of nectar the bees use to trigger their time-memory (i.e. highest sugar concentration of nectar, peak nectar volume, first availability of nectar, or the highest rate of sugar intake).

Question 1: How will changes in water availability affect the squash nectar rhythms for volume and concentration?

Hypothesis 1

The volume and/or sugar concentration of nectar will increase or decrease as the amount of water received by the plant increases or decreases.

In *Primula palinuri*, drought stress has been linked to a reduction in the rate of carbon dioxide up-take leading to a decrease in the production of photosynthate (Dietz and Heber, 1983). Because flowering is resource intensive (Southwick 1984; Galen et al. 1999; Brandenburg et al. 2009), a plant going through periods of low water availability may divert vital resources such as carbohydrates and water away from nectar production in order to maintain the vegetative body of the plant. For example, in *Epilobium angustifolium* (Carroll et al. 2001) a decrease in available water led to a significant decrease in the total volume of nectar produced; unfortunately nectar sugar content was not reported.

A plant experiencing periods of ample water supply will be able to invest more resources into reproduction in order to attract more pollinators. In *Delphinium nelsonii* (Zimmerman 1983) an increase in available water led to higher volumes of nectar when compared to controls, which in turn led to a significant increase in pollinator visits and a higher seed set (fitness). If this is the case for *C.pepo*, we would expect nectar volume and/or concentration to be significantly affected by increases and decreases in available water. Therefore, a simulated drought or rain event will cause the nectar values to deviate from the established rhythms of nectar secretion.

Hypothesis 2

Nectar volume and/or sugar concentration will not be affected by the amount of water received by the plant.

The plant is adapted to secrete the optimal nectar volume and sugar concentration to

attract insect pollinators. Therefore, as the plant experiences changes in available water the nectar rhythms will remain intact in order to maintain reproductive capacity.

Question 2: How do honey bees assess squash nectar quality for time-memory acquisition?

Hypothesis 1

The honey bee time-memory is being triggered by the highest sugar concentration of the nectar.

Honey bees will time their foraging flights on squash flowers to coincide with the period of time when the sucrose concentration is highest, which in turn will maximize foraging efficiency.

Hypothesis 2

Total volume is the component of nectar used by honey bees to trigger their time-memory.

Foragers will show a preference to high volumes of nectar and preferentially arrive when the volume of nectar is highest, which increases the amount of nectar that reaches the hive.

Hypothesis 3

The honey bee time-memory is triggered by the first availability of nectar.

Foragers will time their foraging flights on squash flowers to coincide with the period of time when nectar is initially made available. This allows the honey bee colony to better compete against other potential nectar gatherers.

Hypothesis 4

The honey bee time-memory is formed during the time period with the highest rate of

sugar intake.

Foragers will schedule their foraging flights on squash flowers to coincide with the period of time when the rate of sugar intake is the highest (nectar volume times sugar concentration). In this case, the foragers will optimize foraging efficiency by cueing on the time period of highest sugar intake (molecules of sugar per unit time).

CHAPTER 2

MATERIALS AND METHODS

Study Site 1

Greenhouse experiments (GH1 and GH2) were conducted on the Johnson City campus of East Tennessee State University; the greenhouse is located along West State of Franklin road between Sherrod drive and Lake Street [36°18'18.17"N, 82°22'8.79"W] (see Appendix A). The metal frame greenhouse contains three large benches. The middle bench is flanked by two counters along the interior walls of the structure (see Appendix B).

Greenhouse-Grown Plants

Squash (*Cucurbita pepo* variety 'Summer crookneck') seeds were obtained from the Burpee Seed Company, and planted in soil that was 1 part sand, 5 parts composted manure (Black Kow), and 3B mixture (Fafard). The soil was mixed and two seeds were planted in each 3-gallon plastic pot. Between 2 and 3 weeks after germination the plants were thinned to one plant per pot. Individual pots were numbered and evenly spaced along all three benches of the greenhouse. The use of the greenhouse was preferred as this allowed for controlled conditions as well as excluding possible nectar foragers. Nightly, each plant received the scheduled watering treatment between 1700-2100 hrs.

Greenhouse 1(GH1)

Greenhouse experiment 1 (GH1) was conducted to determine the temporal patterns of nectar secretion for volume, sugar concentration, and sugar production under controlled watering conditions. Three days prior to the start of sampling, and for the entire experiment, a precise

volume of 500 ml of water was delivered to each plant. There were 2 replications of GH1 that consisted of 64 and 100 plants, respectively (Table 1).

Because female and male squash flowers differ in nectar volume and concentration (Nepi et al. 2001) and male flowers were present in such abundance, male blossoms were used exclusively in our study. During anthesis (the time when the flower is open and producing nectar) nectar measurements were taken from 6-10 randomly selected open squash flowers at each sample time. The onset of blossom anthesis varied by 30 min for the 2 trials; different sample times were used for the two replications (Table 1). During each experiment, nectar sampling was conducted every 90 min throughout anthesis. Nectar was extracted from the nectary by 10 μl and 20 μl microcapillary tubes (Drummond Wiretrol). In each sampling, the nectary was completely drained and the flower discarded. The volume of nectar in μl was calculated using the methods described by Kearns and Inouye (1993), the mm of nectar in the pipette was divided by the total length of the tube multiplied by the calibrated volume of the pipette. Once the volume was obtained, a drop of nectar (at least 1μl) was placed on a handheld refractometer (Bellingham + Stanley, Model 45-81) to measure the sugar concentration (w/w) (Corbet et al. 1979). This was done immediately following the volume measurement in order to minimize evaporation of the sample. Due to small volumes in early day extractions, nectar concentration was not measured on some samples. The sugar amount present in each sample was calculated by converting from g/g (from the refractometer reading) to g/mL and multiplying by the density of sucrose as outlined in Kearns and Inouye (1993).

Prior to each sample time, environmental conditions were monitored with a Fisher Enviro-Meter. Light levels (lx), temperature (°C), and relative humidity (%) were recorded at three locations within the greenhouse; these values were used to calculate a mean measurement

for each parameter, establishing the conditions that would be representative of the entire greenhouse. The maximum light level measurable for this instrument is 19000 lux (lx); therefore, light readings that exceeded this value were recorded as 19000 lx. In order to quantify the level of available water in the soil, each day the relative soil saturation value of one plant was taken at random with a soil moisture meter; this took place during the third sample time.

Table 1 Specific information on Greenhouse 1 (GH1) experiments

Experiment number	Dates conducted	Number of plants	Sample period (hrs)
1	7/6/07-7/8/07	64	0500-1230
2	5/19/08-5/21/08	100	0430-1200

Greenhouse 2 (GH2)

Greenhouse experiment 2 (GH2) was conducted to test the impact of available water on the nectar rhythms for volume and sugar concentration. The basic set-up for GH2 involved a period of baseline watering (500 ml), a drought (0 ml), a rain event (1000 ml), and a return back to baseline watering (500 ml). There were two replications of GH2 that consisted of 161 and 183 plants, respectively. The number of days for the baseline and drought periods varied between the two trials (Table 2). In the first run of GH2, the simulated drought was terminated after 9 days, because the plants appeared cracked and badly desiccated. It was unclear how much longer they could survive; therefore, we instituted the simulated rain event. In the second trial of GH2, 10 soil moisture values were taken daily; these values were compared with a one-way ANOVA and the simulated drought was ended once the soil moisture had significantly dropped from pre-drought levels. Throughout anthesis, nectar measurements, and climatic conditions were

recorded as explained above for GH1. Sample times were different between the 2 trials of GH2 (Table 2); however, the sampling interval was kept at 90 min between each pair of sample times.

Table 2 Specific information on Greenhouse 2 (GH2) experiments

Experiment number	Dates conducted	Days of baseline water	Days of drought	Number of plants	Sample period (hrs)
1	9/13/07 - 9/28/07	3	9	161	0430-1200
2	7/17/08 - 8/10/08	4	16	184	0500-1230

Study Site 2

Field experiments (FE 1-3 and BO 1) were conducted on the Kingsport campus of East Tennessee State University, Kingsport, Tennessee [36°33'14.75"N, 82°38'8.54"W], in a grass field, bracketed by a residential area to the south and a forested area to the north. The field was disked and the soil prepared with a rear-tine tiller. Squash (*Cucurbita pepo* variety, 'Summer crookneck') seeds were planted in the soil, augmented with composted manure (Black Kow), and arranged in 8 parallel rows, with approximately 50 plants per row (see appendix C). The rows were spaced 1 m apart and divided into 2 sections based on the order in which they were grown. Rows 1-4 were planted first and rows 5-8 were planted 2 weeks later. To observe foraging behavior in the field, six colonies of honey bees (*Apis mellifera*) were located approximately 150 m north of the squash field along the edge of the tree line. The watering regime was held constant at 4000 ml per plant per day, administered nightly between 1700-2100 hrs.

Field experiments (FE) were conducted to compare with greenhouse experiments as well as to observe honey bee foraging behavior on squash blossoms (Table 3). Bridal veil was used to

prevent insects from visiting designated blossoms (which were chosen at random each day), as it has been shown to affect nectar volume and concentration the least of any bagging technique currently used (Wyatt et al. 1992). The designated buds were chosen and covered the night before sampling took place. Nectar rhythms for volume, sugar concentration, and sugar production for both veiled (which were compared with greenhouse rhythms) and unveiled flowers were discovered. Furthermore, we observed what time in anthesis the honey bees had triggered their time-memory. The initial field experiments (FE 1 and 2) were conducted on rows 1-4, as they began to flower first. Throughout anthesis, nectar measurements were recorded as explained above for the greenhouse experiments, with the addition of 8-10 veiled flowers also taken at each sample time. The interval between sample times was held constant at 90 min. In FE 1, sampling began at 0600 and concluded at 1200 hrs.

Observations in the field were made at three different observation posts. The locations were selected based on having a roughly equal density of male flowers present on the first day of the experiment. The observer was seated facing the observation area (a 2-M section of row) and recorded the time and number of honey bee arrivals seen within the observation area. At each station observations were made for 20 minutes every hour on the hour beginning at sunrise and concluding at sunset. The times for sunrise and sunset were obtained from Weather Underground (2008).

After following the above procedure for 3 consecutive days, a test day was performed in which all the flowers in the field were removed. During the test day, the squash field was monitored for honey bee visits as in the previous 3 days. The removal of the flowers was necessary in order to observe the temporal distribution of arrivals anticipating a food reward. The time-memory was determined to have been formed during the time period in which the

greatest number of arrivals was observed on the test day.

Climatic conditions, light levels (lx), temperature (°C), and relative humidity (%), were recorded prior to each sample time with a Fisher Enviro-Meter. Readings were taken at the edge of the squash field.

The second trial of FE was modified in order to more accurately determine what honey bee foragers were collecting in the field: nectar, pollen, or both. The number of observation stations was increased from 3 to 5. Also, nectar sampling and bee observation times (0730, 0900, and 1030 hrs) were more narrowly focused on the period of time of peak honey bee foraging activity. A population coding system was set in place in which 15 minutes prior to and after each 30 minute observation time observers would place a dab of Testors paint on the abdomen of every forager present, indicating what sample time she was observed. A painted bee that arrived at a different sample time from the one in which she was originally seen and painted was re-painted with a different color to denote which sample times she was observed. This color coding system allowed the observers to determine if individual foraging subgroups were restricted to certain time periods in anthesis or if they were foraging during multiple sample times.

During the observation times, each forager's color, arrival time, and foraging behavior were recorded. A forager that only probed the nectary and had no pollen on her pollen baskets was regarded as a nectar forager (N). A bee that was seen visiting the anther and the nectary or probed the nectary with pollen in her pollen baskets was referred to as a nectar and pollen forager (NP). Foragers that visited the anther exclusively or had pollen in their pollen baskets and did not investigate the nectary was regarded as a pollen forager (P). If the foraging behavior was unknown, as the bee had no pollen in her baskets and/or she did not visit either the anther or

the nectary, the foraging behavior was undetermined and the bee received a question mark (?).

Nectar measurements were taken as explained above for FE 1 along with flower anthers that were also collected during anthesis. Pollen levels in the field were monitored by the collection of 10 anthers from veiled flowers at 0630 and 1030 hrs as controls and from unveiled flowers at 0730, 0900 and 1030 hrs. The controls were collected in order to quantify the pollen movement in the field as well as to quantify possible environmental variables such as the effect of wind on pollen levels on the anthers. The above procedure was followed for 3 days after which a test day was performed. During the test day the number of observation posts increased from 5 to 7; they were monitored for 30 min at each sample time (0745, 0915, and 1045).

Pollen counts were made later in the lab using the following technique (Mike Zavada, *personal communication*): each anther was submerged in 10 ml of DI water and macerated with a glass stirring rod. One drop of Tween 80 and Saffron red stain were added. After mixing, a pipette was used to transfer 0.1 ml to a glass slide, and a cover slip was placed on top. From each anther collected, three slides were made using the above technique. Using a compound microscope, the number of pollen grains was counted for each slide and multiplied by 100 to give the number of pollen grains per 10 ml of solution. From each anther, the three slides were used to calculate a mean pollen count.

In the third trial of FE only pollen and nectar samples were collected; this was done on rows 5-8 of the squash field. Nectar samples were collected as stated above every 90 min from 0600-1200 hrs. Seven anthers were collected at each sample time 0600, 0730, 0900, 1030 and 1200 hrs, with 7 veiled anthers taken at 0600 hrs and 1200 hrs to serve as a control.

To further quantify honey bee and bumble bee (*Bombus sp*) pollinator foraging activity, the Bee observation experiments (BO) were performed. In these experiments the observer

conducted a slow observation walk through rows 5-8 of the squash field; these were conducted every hour on the hour from 0600-1700 hrs. For a period of 20 min the observer would note the number of honey bees and bumble bees seen at each sample time. It was determined that during an observation walk, the observer could only accurately keep track of one side of a row of squash at a time. Therefore, each side of each row was given a number from 1 to 8. To prevent observer bias, a list of randomly generated numbers with repeats from 1 to 8 was used to randomize the order of the observation walk.

Table 3 Specific information on field experiments (FE)

Experiment name and number	Dates conducted	Squash rows used	Nectar sampling period	Number of observation posts	Number of observation posts on test day	Observation period	Pollen sample period
FE 1	8/11/08 - 8/14/08	1-4	0600 - 1200	3	3	0600 - 2000	N/A
FE 2	8/20/08 - 8/23/08	1-4	0730 - 1030	5	7	0730 - 1030	0630 - 1030
FE 3	9/3/08 - 9/5/08	5-8	0600 - 1200	N/A	N/A	N/A	0600 - 1200

Data Analysis (GH1,GH2, FE 1-3, BO 1)

The nectar volume, sugar concentration, and the amount of sugar produced were analyzed as dependent variables with sample time and experimental day as treatment effects using Analysis of variance (ANOVA). Using a two-way ANOVA model we investigated: (1) the relationship between concentration, sample time, and experimental day, (2) the relationship between volume, sample time, and experimental day, and (3) the relationship between sugar amount, sample time, and experimental day. In this analysis experimental day was used to represent watering amount. Significant findings were further analyzed using the Tukey post hoc

test which uses a pair wise comparison of means. Calculations were done using either Microsoft Excel or the statistical software package SPSS 16. One-way ANOVAs were run on the relative soil saturation values, pollen counts, and test day arrivals (FE 2) followed by Tukey post hoc analyses.

CHAPTER 3

RESULTS

Experiments involving nectar measurements (GH1, GH2, and FE 1-3) were analyzed separately, yielding similar results. This was done because the onset time of flower anthesis varied between experiments as did collection times. Furthermore, in each experiment a square root transformation of the dependent variables volume and sugar amount were employed to give normally distributed residuals (see Appendix D).

In all nectar experiments with the exception of FE 1 the climatic data (temperature, humidity, and light levels) were not included as they did not improve the accuracy of the statistical models. The variability explained by these independent variables was already contained in sample time. In FE 1, the independent variable temperature was found to be significant in predicting sugar amount, which may be due to normal variation in nectar secretion or possibly sampling error.

Nectar sugar secretion rate (mg/hr) for each sample period was determined for GH1 B, GH2 B, and FE 1 experiments. The rate of secretion was found using the difference in the mean amount of sugar (mg) present between two sample times and converting to mg/hr. Due to the unequal sample sizes between the pair of sample times, sugar secretion rates were determined using means; therefore, statistical tests were not employed.

GH1 Experiments

The baseline experiments (GH1) demonstrated significant diel trends in nectar production for volume, concentration, and sugar amount. Analyzed separately, the results are similar for

both trials of GH1. Sample time (time of day) was significant in determining nectar volume, concentration, and sugar amount ($p < 0.001$) but experimental day was not ($p > 0.05$ for all trials, Table 4). These results indicate that experimental day (watering amount) was not a significant predictor of nectar volume, concentration, or sugar amount.

Table 4 P-values from ANOVA analysis for GH1 experiments

GH1 A		
	Sample time	**Experimental day**
Volume	$p < 0.001$	$p > 0.05$
Concentration	$p < 0.001$	$p > 0.05$
Sugar	$p < 0.001$	$P > 0.05$

GH1 B		
	Sample time	**Experimental day**
Volume	$p < 0.001$	$p > 0.05$
Concentration	$p < 0.001$	$p > 0.05$
Sugar	$p < 0.001$	$p > 0.05$

The dependent variables volume and sugar amount went through square root transformations. Experiments conducted 7/6/07-7/8/07 and 5/19/08-5/25/08.

Figure 1 shows mean nectar volume, concentration, and sugar amount by time of day for days 1-3 of the second trial of GH1. Nectar volume shows an increase from blossom opening to closing. Post hoc analysis shows that sample times 1 and 2 are not significantly different from each other. However, they are significantly different from sample times 3, 4, 5, and 6 ($p < 0.001$). Sugar concentration decreased from flower opening to closing. The Tukey post hoc analysis showed that when looking at concentration by sample time values for sample times 1 and 2 are not significantly different from each other; however, they are significantly different from sample times 3, 4, 5, and 6 ($p < 0.01$). Sugar amount (mg) shows a significant increase from blossom opening to closing. The Tukey post hoc analysis shows that sugar values at sample times 1 and 2 are not significantly different from each other; however, they are significantly different from sample times 3, 4, 5, and 6 ($p < 0.05$).

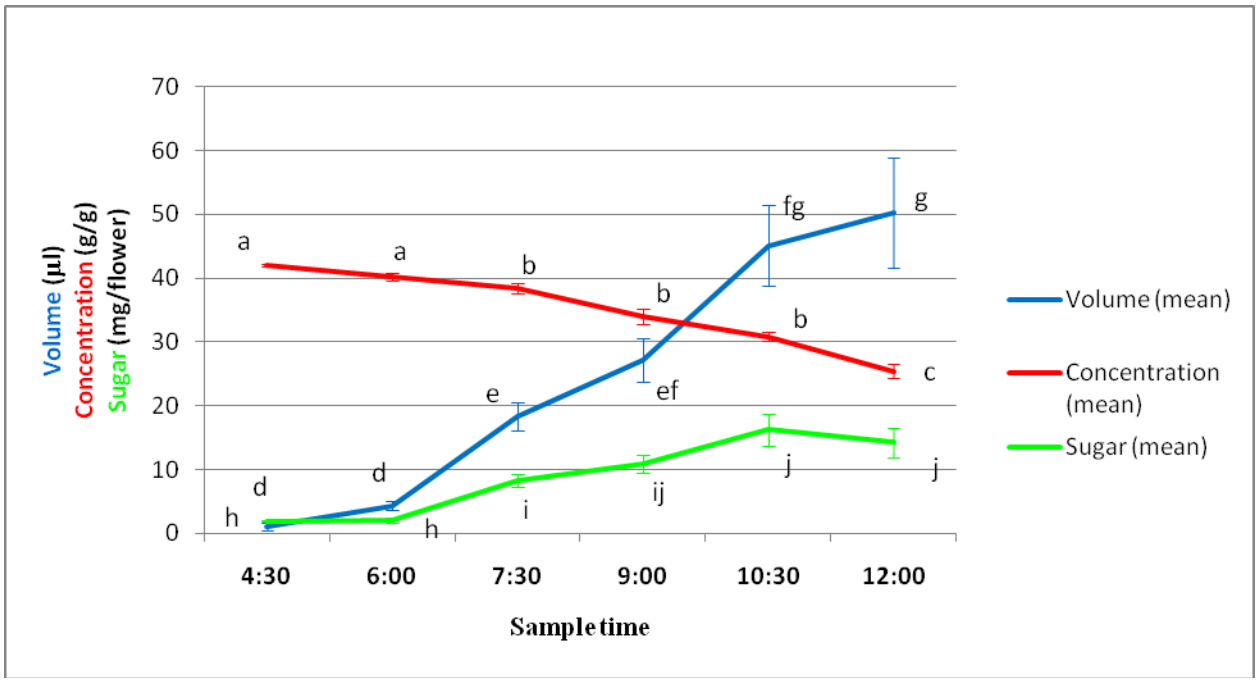


Figure 1 Nectar attributes for Greenhouse 1 (GH1 B). Experiment conducted from May 19th through May 21st 2008. Nectar volume (blue line) (n = 16 for 4:30, 6:00, 7:30, 9:00, and 10:30, 11 for 12:00), sugar concentration (red line) (n = 4 for 4:30, 18 for 6:00, 19 for 7:30, 18 for 9:00, 19 for 10:30, and 11 for 12:00), and sugar amount (green line) (n = 4 for 4:30, 18 for 6:00, 19 for 7:30, 18 for 9:00, 19 for 10:30, and 11 for 12:00) per flower (mean $\pm$ s.e.m.). Baseline watering conditions were applied. Data points are significantly different if they do not share a common letter. The significance level for volume and concentration is $p < 0.001$. Study conducted in the small greenhouse located on the main campus of ETSU.

Figure 2 shows the mean nectar sugar secretion rate per time period per flower for GH1 B. Although no statistical test was performed, it appears as though the rate of sugar secretion varies by time of day. The sugar secretion rate appears to be highest from 0630 to 1030 hrs, peaking at 4.16 mg/hr from 0600 to 0730 hrs. The negative value reported (1030 to 1200 hrs) may be due to either sampling error or possibly reabsorption of nectar, which has been empirically demonstrated in *C.pepo* (Nepi et al. 2001).

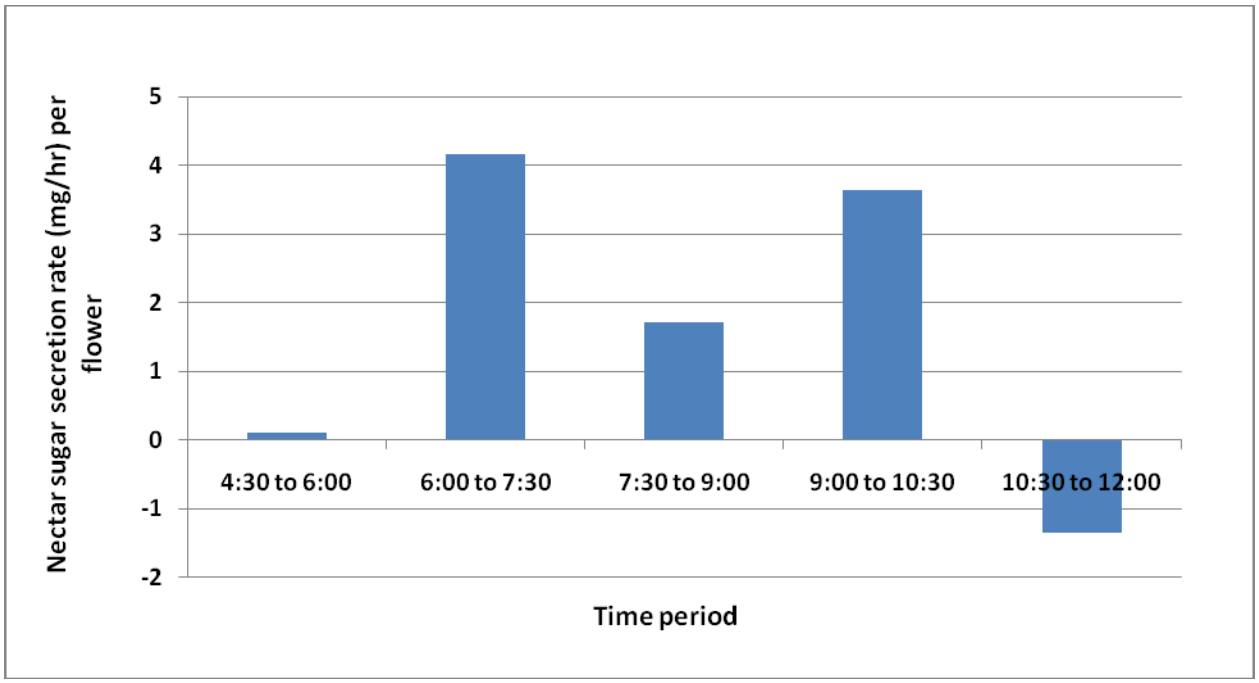


Figure 2 Mean nectar sugar secretion rate (mg/hr) per flower for GH1 B

GH2 Experiments

Despite simulated droughts of different lengths, the statistical analysis provided similar results in both trials of GH2. Both sample time and experimental day were significant in predicting nectar volume and sugar concentration ($p < 0.01$ for all trials, Table 5). Sample time was significant in predicting sugar amount in both trials ($p < 0.001$); however, experimental day was only significant in GH2 B ($p < 0.001$).

Table 5 P-values from ANOVA analysis for GH2 experiments

GH2 A		
	Sample time	**Experimental day**
Volume	$p < 0.001$	$p < 0.001$
Concentration	$p < 0.001$	$p < 0.001$
Sugar	$p < 0.001$	$p > 0.05$

GH2 B		
	Sample time	**Experimental day**
Volume	$p < 0.001$	$p < 0.01$
Concentration	$p < 0.001$	$p < 0.001$
Sugar	$p < 0.001$	$p < 0.001$

The dependent variables volume and sugar amount went through square root transformations. Experiments conducted 9/13/07-9/28/07 and 7/17/08-8/10/08.

In GH2 B the moisture content of the soil showed a gradual but significant decline during the simulated drought, which occurred from days 5-20 (Figure 3 A). It was not until day 12, which was 8 days into the drought, that the mean soil moisture level significantly dropped from pre-drought conditions. The Tukey post hoc analysis of a one-way ANOVA run on relative soil saturation values found that days 1-4 (baseline), days 17-20 (late drought), and days 24-25 (post drought) are all significantly different from each other ($p < 0.001$). These results demonstrate that the soil moisture decreased significantly during the simulated drought, confirming that the plants were exposed to a period of lower water availability.

It took a number of days for the soil moisture to begin to rebound after the plants began to once again receive water. This was probably because the soil was extremely dry. In addition, the soil moisture level was never significantly higher than baseline conditions.

In GH2 B, experimental day (watering amount) was significant; fluctuations are seen in the amplitude of the nectar rhythms; however, the overall pattern is consistent from day to day (Figure 3 B). Sugar concentration demonstrated consistent and significant temporal trends in

days 1 ($p < 0.001$), 13 ($p < 0.001$), and 25 ($p < 0.001$), with values starting high early on and decreasing through anthesis. On the other hand, volume showed significant diel trends in days 1 ($p < 0.001$), and 13 ($p < 0.001$), with volume increasing through anthesis. In day 25, a similar significant trend in volume secretion was seen ($p < 0.001$), with a non-significant decrease in volume observed from 0930 hrs to 1100 hrs, which may be attributed to sampling error. Sugar amount shows a significant increase from blossom opening to closing in day 1 ($p < 0.001$) and 13 ($p <0.001$) but not in day 25 ($p > 0.05$), possibly due to sampling error or random fluctuations in nectar secretion.

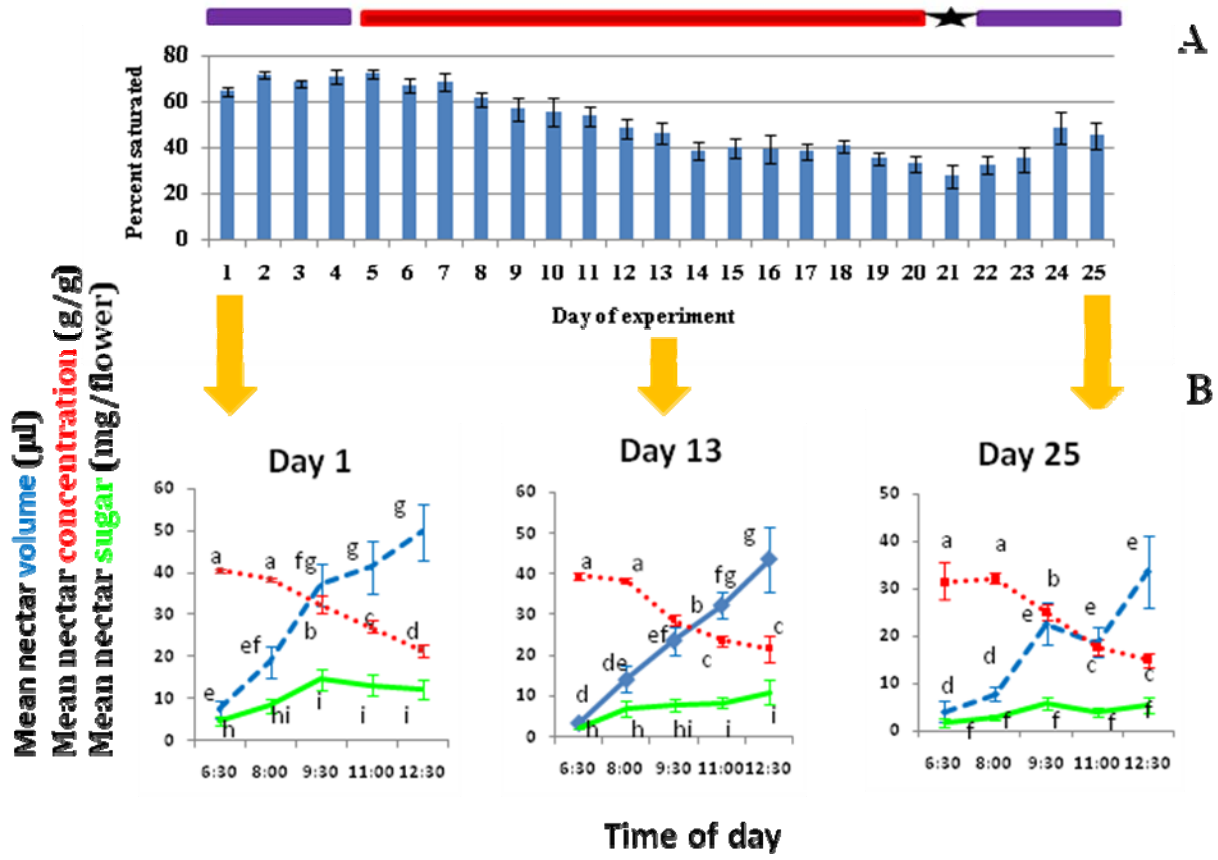


Figure 3 Greenhouse 2 (GH2 B) conducted from July 17th to Auguest 10th 2008. (A) The mean relative soil saturation values (± s.e.m.) of GH2 B. Each day during the 0930 sample time, 10 plants were sampled. The symbols above the days represent watering regime each plant recieved: baseline of 500 ml (purple line), drought with no water (red line), and the rain event with 1000 ml (star). (B) Mean nectar volume (dashed line), sugar concentration (dotted line), and sugar amount (solid line) per flower (± s.e.m.) for days 1, 13, and 25 of GH2 B. Day 1 nectar volume (n = 12 for 6:30, 8:00, 10 for 9:30, 11:00, and 9 for 12:30). Day 1 nectar concentration (n = 9 for 6:30, 12 for 8:00, 10 for 9:30, 11:00, 9 for 12:30). Day 1 sugar amount (n = 9 for 6:30, 12 for 8:00, 10 for 9:30, 11:00, 9 for 12:30). Day 13 nectar volume (n = 12 for 6:30, 8:00, 10 for 9:30, 11:00, and 4 for 12:30). Day 13 nectar concentration (n = 8 for 6:30, 11 for 8:00, 12 for 9:30, 10 for 11:00, and 4 for 12:30).). Day 13 sugar amount (n = 8 for 6:30, 11 for 8:00, 12 for 9:30, 10 for 11:00, and 4 for 12:30). Day 25 nectar volume (n = 9 for 6:30, 8:00, 10 for 9:30, 11:00, and 5 for 12:30). Day 25 nectar concentration (n = 4 for 6:30, 9 for 8:00, 8 for 9:30, 10 for 11:00, and 5 for 12:30). Day 25 sugar amount (n = 6 for 6:30, 9 for 8:00, 9 for 9:30, 10 for 11:00, and 5 for 12:30). Data points are significantly different if they do not share a common letter. The significance level for volume, concentration, and sugar amount is $p < 0.001$.

Figure 4 shows the mean nectar sugar secretion rate (mg/hr) per flower for days 1, 13, and 25 of GH2 B. Although there is no clear diurnal pattern from day to day, in all 3 days (1, 13, and 25) it appears as though the secretion rate peaks between 0630 and 0930 hrs. As stated previously, the negative values reported may be due to sampling error or reabsorption.

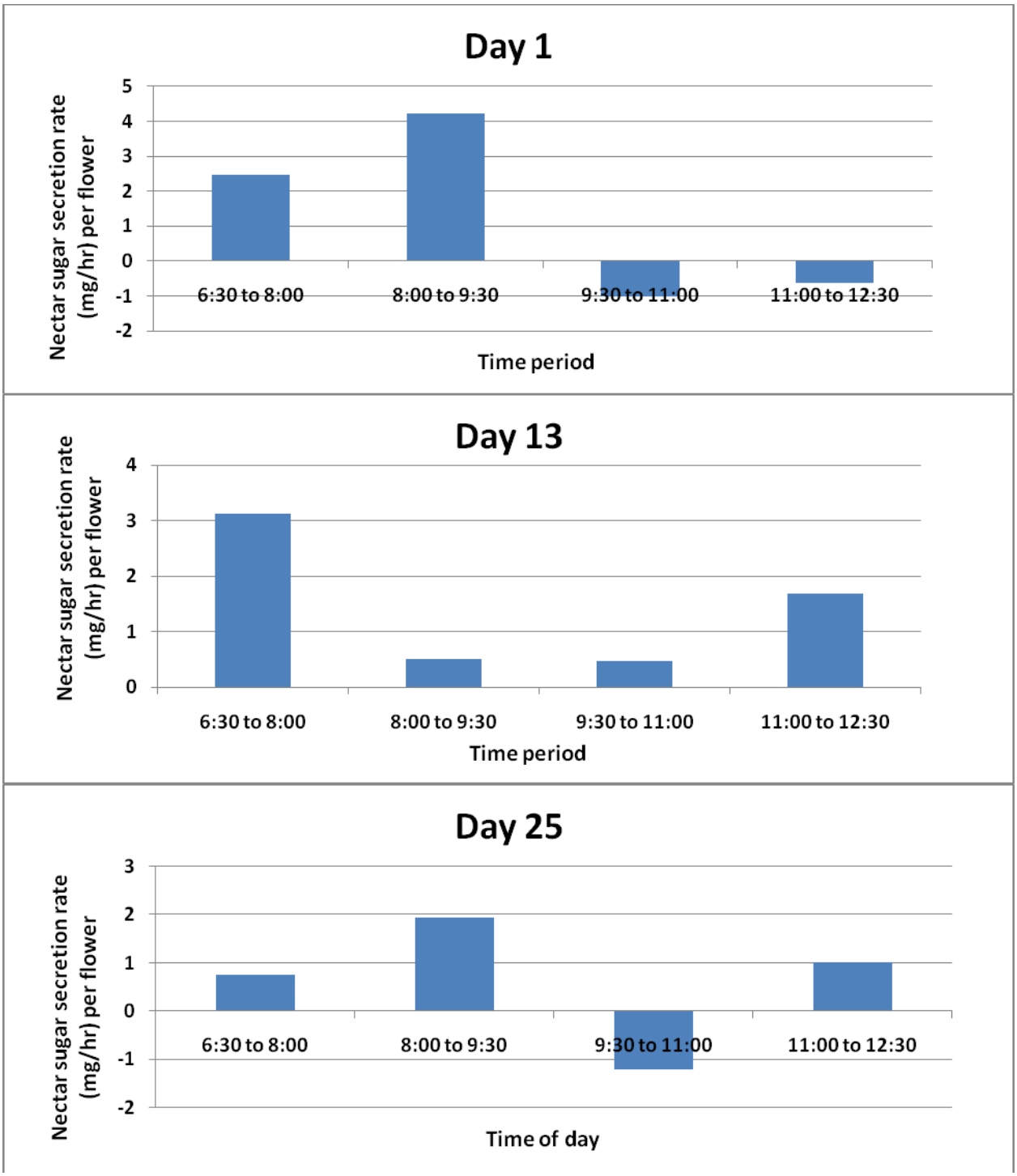


Figure 4 Mean nectar sugar production (mg/hr) per flower for days 1, 13, and 25 of GH2 B

Field Experiments

The standing crop of the unveiled flowers was less than 1 μl at each sample time, most likely due to the inundation of bumble bees (*Bombus* sp.) seen in our field experiments (FE 1 and FE 2). Therefore, due to low nectar volumes, we were unable to obtain accurate nectar readings for unveiled flowers throughout anthesis.

The field experiments (FE 1-3) showed consistent diel trends in nectar secretion for veiled flowers as demonstrated in FE 1 (Figure 5). Sample time was significant in predicting nectar volume, sugar concentration, and sugar amount for all trials ($p < 0.001$, Table 6). The veiled flowers showed consistent diel trends that were very similar to the rhythms established in the greenhouse. In FE 1 nectar volume and sugar amount significantly increased throughout the day (Figure 5, $P < 0.001$). Post hoc analysis showed that for volume sample times 1 and 2 are significantly different from each other as well as sample times 3, 4, and 5 ($p < 0.001$). Sugar amount by sample time shows that according to post hoc analysis values at sample times 1 and 2 are not significantly different from each other; however, they are significantly different from sample times 3, 4, and 5 ($p < 0.001$). On the other hand, the sugar concentration showed a decrease throughout the day. The Tukey post hoc analysis showed that for sugar concentration, sample times 1, 2, 3, and 4 are not significantly different from each other; however, they are significantly different from sample time 5 ($p < 0.001$).

Experimental day was a significant predictor of volume and sugar amount for both FE 1 and FE 3. The significance of experimental day in FE 1 and FE 3 may have been due to normal variation in nectar secretion. These fluctuations would be more pronounced as both FE 1 and FE 3 experiments had a number of sample times that suffered from low sample sizes, 6-8, compared to FE 2, in which 12 flowers were taken at each sample time.

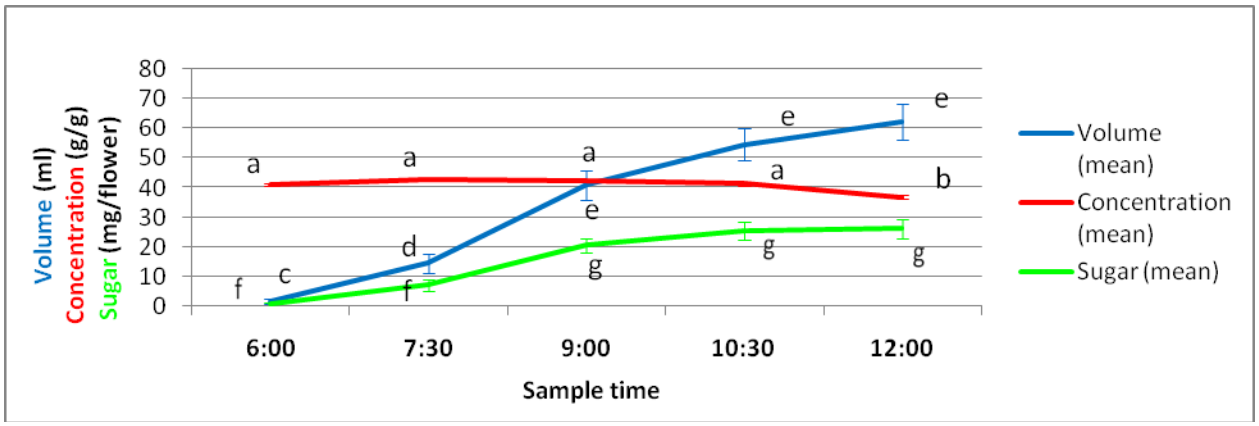


Figure 5 Nectar attributes for FE 1. Nectar volume (blue line) (n = 26 for 6:00, 24 for 7:30, 9:00, and 10:30, 17 for 12:00), sugar concentration (red line) (n = 7 for 6:00, 16 for 7:30, 23 for 9:00, 10:30, and 16 for 12:00), and sugar amount (green line) (n = 7 for 6:00, 16 for 7:30, 23 for 9:00, 10:30, and 16 for 12:00) per flower (mean ± s.e.m.). Data points are significantly different if they do not share a common letter. Study was conducted in Kingsport TN (8/11/08 – 8/13/08).

Figure 6 shows the mean nectar sugar secretion rate (mg/hr) per flower for FE 1. The sugar secretion rate appears to peak from 0730 to 0900 hrs, at 8.94 mg/hr, with the sugar secretion rate decreasing each sample time from 0900 to 1200.

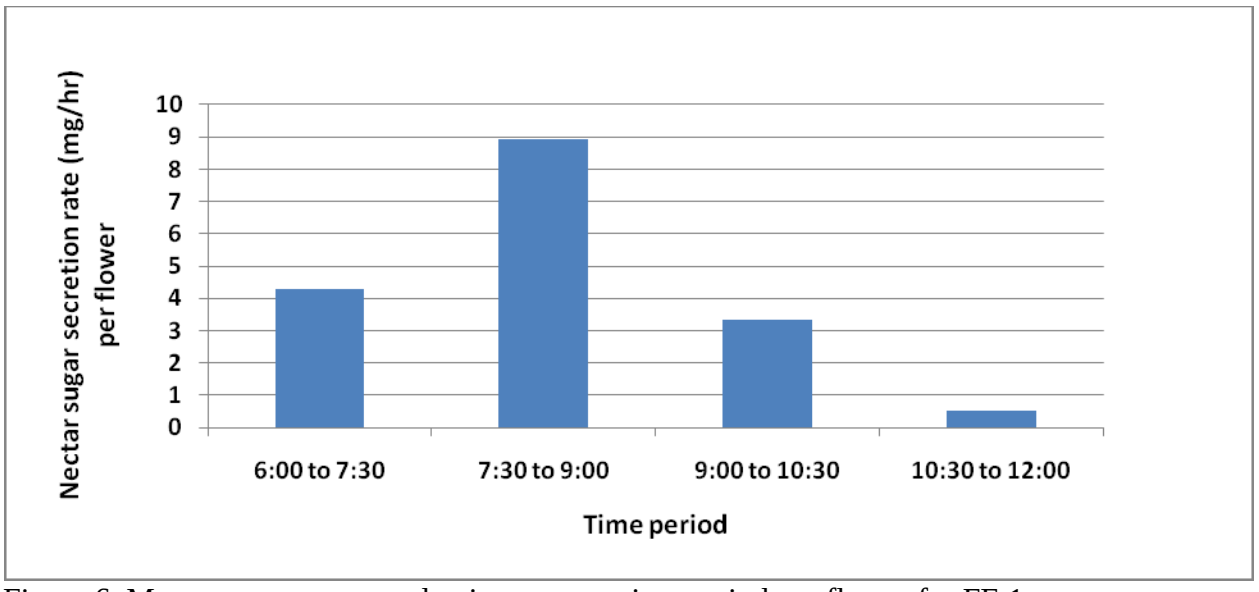


Figure 6 Mean nectar sugar production rate per time period per flower for FE 1

Table 6. P-values from ANOVA analysis for field experiments (FE 1-3)

FE (1)		
	Sample time	**Experimental day**
Volume	$p < 0.001$	$p < 0.001$
Concentration	$p < 0.001$	$p > 0.05$
Sugar	$p < 0.001$	$p < 0.05$

FE (2)		
	Sample time	**Experimental day**
Volume	$p < 0.001$	$p > 0.05$
Concentration	$p < 0.001$	$p > 0.05$
Sugar	$p < 0.001$	$p > 0.05$

FE (3)		
	Sample time	**Experimental day**
Volume	$p < 0.001$	$p < 0.01$
Concentration	$p < 0.001$	$p > 0.05$
Sugar	$p < 0.001$	$p < 0.05$

The dependent variable volume went through a square root transformation. Experiments conducted: 8/11/08-8/13/08, 8/20/08-8/22/08, 9/3/08-9/5/08.

Honey bee foraging activity in the field was restricted to the hours of anthesis (Figures 7-8). In FE 1, honey bee arrivals to the squash field started just after sunrise (0644 hrs) with 5.11 $\pm$ 0.11 (mean $\pm$ s.e.m.) arrivals seen during the 0700 sample time (Figure 7). Arrivals were seen throughout anthesis, peaking between 0800 and 0900 hrs, with 10.33 $\pm$ 0.88 and 10.11 $\pm$ 1.55 arrivals (mean $\pm$ s.e.m.), respectively. With no floral rewards available, the number of arrivals quickly diminished after the flowers closed around 1400 hrs. Consequently, honey bees were almost completely absent from the field from 1500 to 2000 hrs.

The pattern of honey bee arrivals seen in BO 1 was similar to what was seen in FE 1 (Figure 8). Honey bees arrived to the squash blossoms slightly later (0800 hrs) because sunrise was also later (0714 hrs). Foraging honey bees were seen throughout anthesis, peaking at 1000

hrs with 10.0 ± 0.50 (mean ± s.e.m.). Honey bees were almost completely absent from the field after the flowers had closed around 1300 hrs. Bumble bee activity started earlier (0700 hrs) and ended later (1300 hrs) than honey bee activity, with peak activity seen at 1100 hrs with 15.0 ± 9.0 arrivals (mean ± s.e.m.).

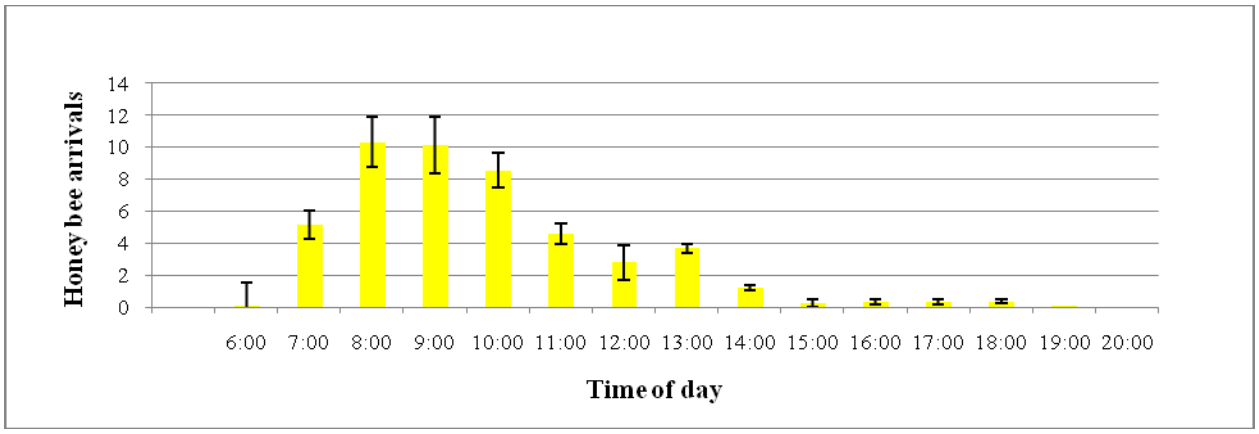


Figure 7 Average (± s.e.m.) honey bee arrivals per patch per day for FE 1. Three patches were monitored every hour on the hour for 20 minutes (8/11/08 – 8/13/08); conducted in Kingsport, Tennessee (sunrise 0644 hrs, sunset 2024 hrs).

In FE 2, the number of honey bee arrivals (both marked and unmarked combined) started off slowly with 1.93 ± 0.46 (mean ± s.e.m.) observed during the 0730 sample time. Foraging activity increased by sample time, peaking at 9.73 ± 1.69 (mean ± s.e.m.) during the 1030 sample time.

The marked cohorts in Figure 9 reveal that the number of arrivals for each colored cohort varied by sample time. The number of white marked bees goes from 0.07 ± 0.07 (mean ± s.e.m.) at 0730 to 1.33 ± 0.39 (mean ± s.e.m.) at 0900 hrs before peaking during the 1030 sample time with 2.67 ± 0.43 (mean ± s.e.m.). This general increase in arrivals by sample time was seen in blue and yellow cohorts as well.

Because the actual number of bees contained in each cohort varied from day to day and from cohort to cohort, there were no direct comparisions made. However, it is clear that each

colored cohort of bees was observed at multiple sample times. This illustrates that these foragering subgroups were not restricted to a certain sample time but were observed throughout anthesis.

The honey bees (marked and umarked combined) in FE 2 were observed preferentially collecting nectar. At both the 0900 and 1030 sample times, foragers were observed gathering nectar (N) in preference to pollen (P) or pollen and nectar (NP) (Figure 10). There were 4.33 $\pm$ 2.26 and 7.53 $\pm$ 2.08 nectar foragers (mean $\pm$ s.e.m.) observed during the 0900 and 1030 sample times, respectively.

Pollen levels in the field start fairly high with 32,581 $\pm$ 3,286 (mean $\pm$ s.e.m.) pollen grains per anther found at the 0630 sample time (Figure 11). However, the decline in pollen is rapid and significant reaching 505 $\pm$ 0.11 (mean $\pm$ s.e.m.) pollen grains per anther by the 1030 sample time. Little pollen was available to the honey bees during the time of their peak foraging activity (0900 and 1030 sample times). Therefore, the pollen movement in the field must be atrributed to other pollinator activity such as the ubiquitous foraging activity of the bumble bees (*Bombus* sp.) that were present in the field.

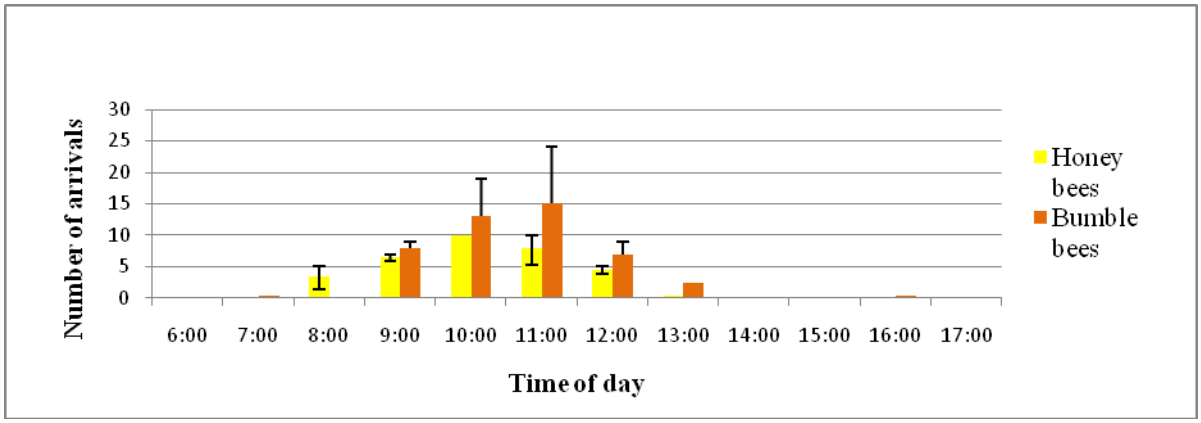


Figure 8 Average number (± s.e.m.) of honey bee and bumble bee arrivals for BO 1. Observations occurred every hour on the hour for 20 min, with the observer taking a slow walk through the field, noting the number of honey bees and bumble bees encountered (9/18/08 – 9/19/08); Conducted in Kingsport, Tennessee (sunrise 0714 hrs, sunset 1931 hrs).

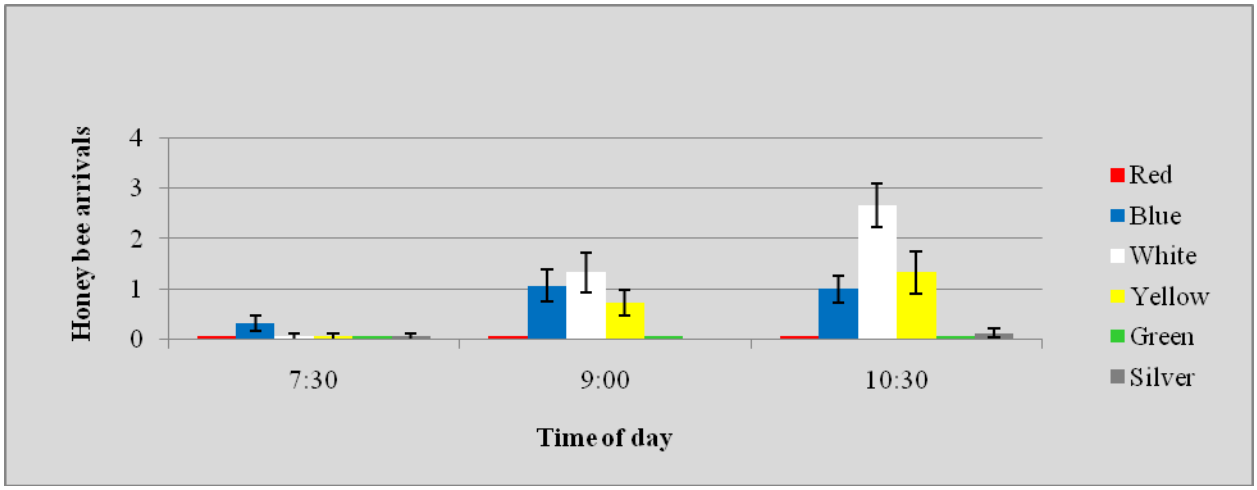


Figure 9 The mean number (± s.e.m.) of marked honey bee arrivals for FE 2. Five stations were monitored for 30 min periods at each sample time for 3 consecutive days (8/20/08 – 8/22/08). The experiment was conducted in Kingsport, Tennessee (sunrise 0651hrs, sunset 2013 hrs).

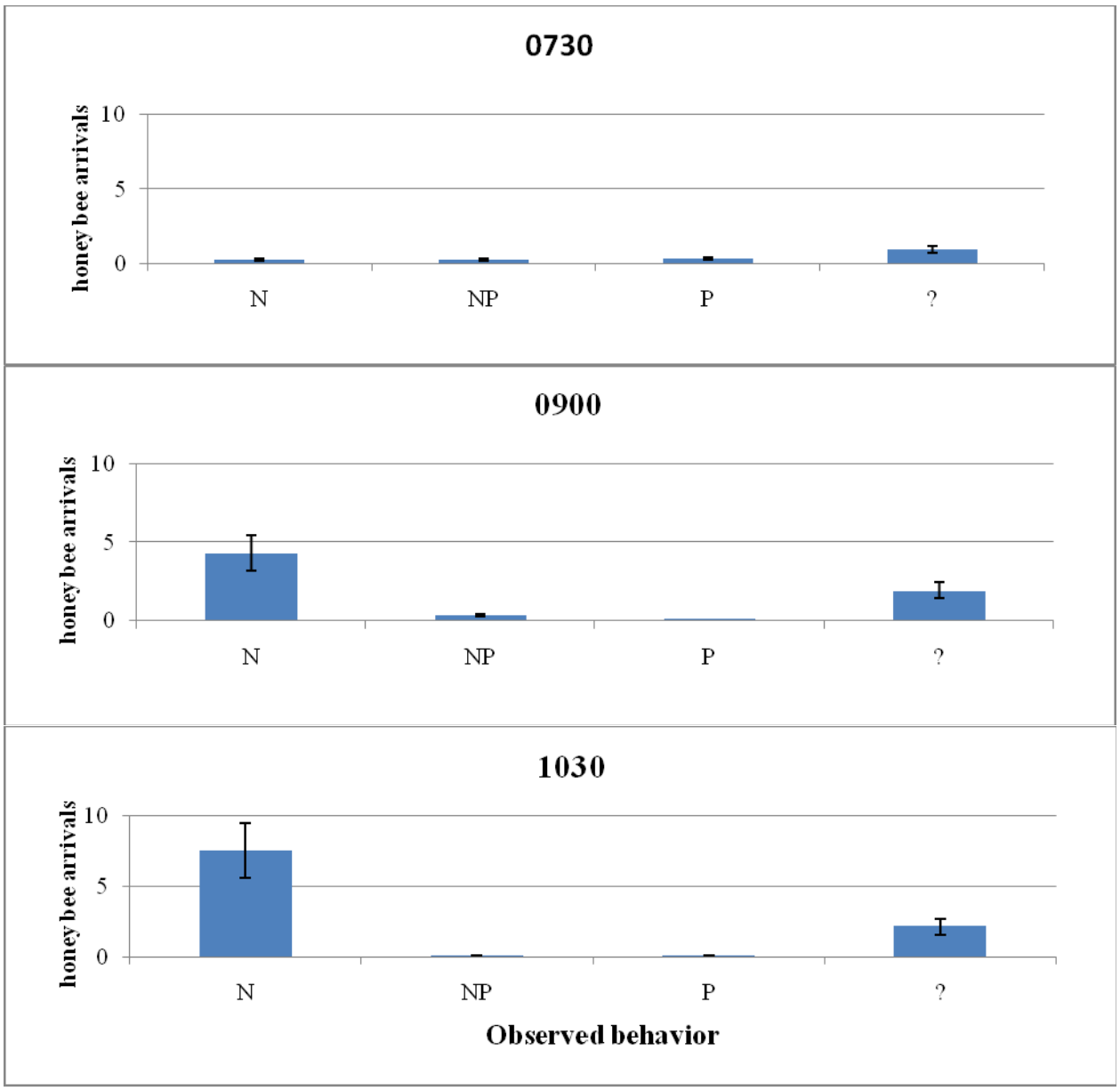


Figure 10 The mean number (± s.e.m.) of honey bee foragers by time of day in FE 2. Foraging behavior observed: nectar (N), nectar and pollen (NP), pollen (P) or undetermined (?). Five stations were monitored for 30 min periods at each sample time. The experiment was conducted in Kingsport, Tennessee (8/20/08 – 8/22/08) (sunrise 0651hrs, sunset 2013 hrs).

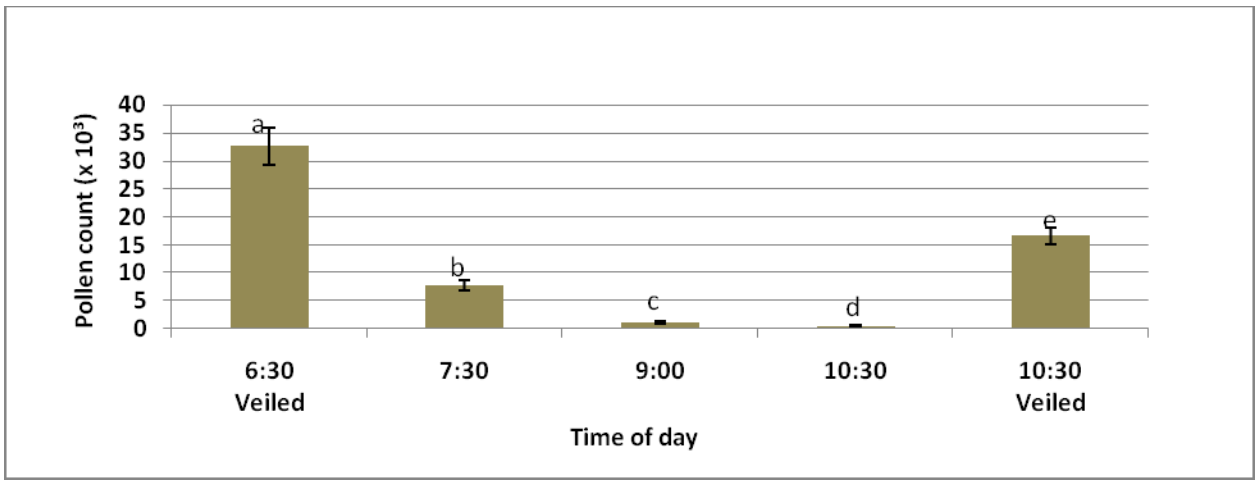


Figure 11 Pollen counts per anther (mean ± s.e.m.) for FE 2. For 3 consecutive days, 10 anthers were collected at each of five sample times (8/20/08 – 8/22/08). Pollen counts were performed later in the lab. Data are significantly different if they do not share a common letter. The significance level for pollen is $p < 0.001$.

In FE 1 and FE 2, there was a stark difference in the arrival pattern of honey bees observed during each test day (Figures 12 and 13). For FE 1, arrivals began early, however, they do not peak until the 1100 sample time (Figure 12). During the observation days for FE 1 (Figure 7), the peak activity was a 2 hr window from 0800-1000 hrs, which was the period in which very few arrivals were seen on the test day. It was during this 2 hr period that heavy fog enveloped the field disrupting foraging flights, as honey bees navigate using the sun as a compass (von Frisch 1967). In FE 2, the number of arrivals peaked early and decreased during each subsequent sample time (Figure 13). A one-way ANOVA showed that the arrival numbers at each sample time (0745, 0915, and 1045) were not significantly different from each other.

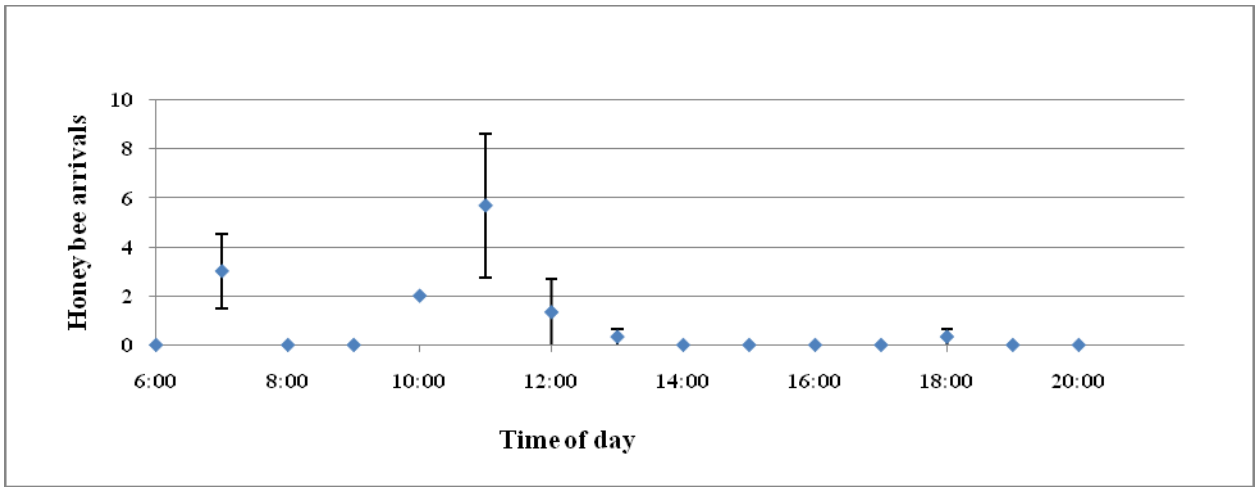


Figure 12 Number of honey bee arrivals (mean ± s.e.m.) observed on the test day of FE 1. Three stations were observed every hour on the hour for 20 minutes. Heavy fog was observed during the 0800 and 0900 sample times.

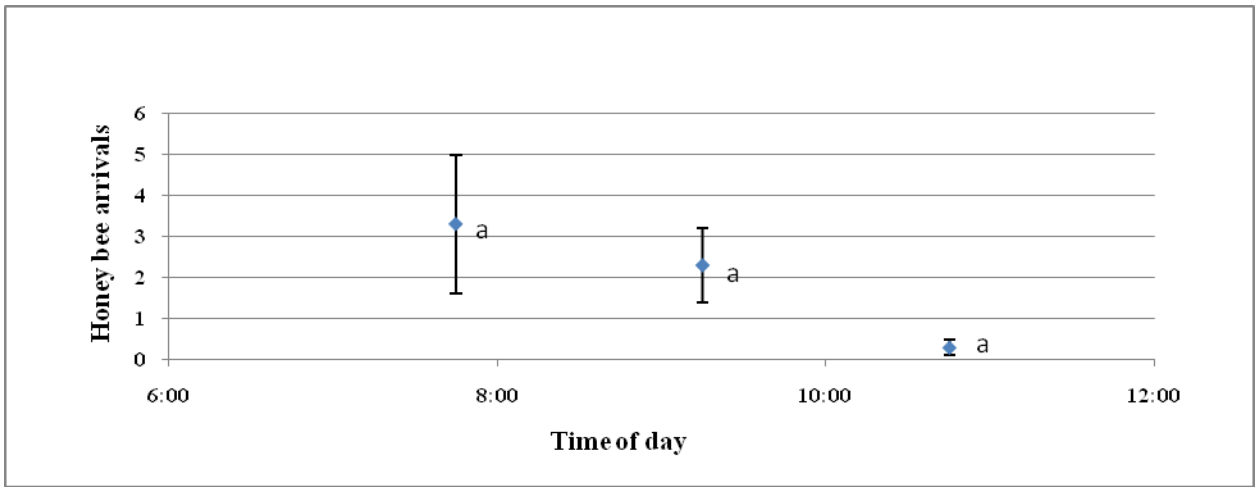


Figure 13 Number of honey bee arrivals (mean ± s.e.m.) observed on the test day of FE 2. Data points are significantly different if they do not share a common letter. Seven stations were observed at 0745, 0915 and 1045 hrs for a period of 30 minutes.

CHAPTER 4

DISCUSSION

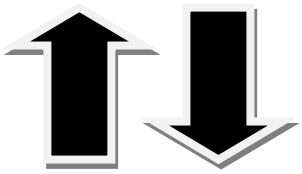
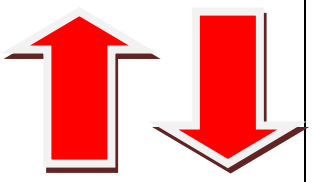
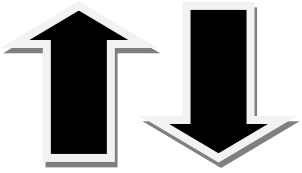
Accounting for multiple factors (genetic variability, gender bias, and within plant variation) has enabled us to present an accurate description of the diel pattern of nectar secretion in *C.pepo*. Despite fluctuations in water availability and daily variation in climate, these trends in nectar secretion were shown to be remarkably consistent. Plants grown both indoors and outdoors demonstrated similar nectar rhythms. Our research describes a robust rhythm of nectar secretion for *C.pepo* that shows a diel increase in nectar volume and sugar amount that is accompanied by a decrease in sugar concentration during the period in which the flowers are open and available to pollinators. In our field studies, we describe an increase in volume from blossom opening until 0900 hrs, which is in agreement with previous work (Nepi et al. 2001). However, Nepi and his colleagues found nectar concentration did not alter significantly as an effect of time. We found that concentration (g/g) decreased significantly from 1030 to 1200 hrs. This discrepancy may be due to the larger sample size in our study, allowing for detection of smaller fluctuations in nectar concentration or genetic variation as they used *C. pepo* 'Greyzini' in their study.

Table 7 illustrates the possible relationship between water availability and nectar secretion (volume and sugar concentration) in squash blossoms. Hypotheses 1 proposes that fluctuations in the amount of water available to the plant will affect the volume and/or the sugar concentration of the nectar produced. An increase or decrease in available water will cause a significant increase or decrease in nectar volume and/or sugar concentration, leading to a significant change in the diel trend of nectar secretion. On the other hand, Hypothesis 2 proposes

that nectar volume and concentration will not be significantly affected by fluctuations in available water; the rhythms of nectar secretion will persist.

In the second trial of GH2 the relative soil saturation values show that the plants did experience a period of low water availability that was significantly lower than pre-drought conditions (Figure 3 A). However, the plants did not experience a period of soil moisture significantly higher than baseline levels. These results indicate that hypothesis 1 and 2 were only partially tested because the plants did experience a drought period.

Table 7 The possible relationship between water availability and nectar secretion in squash

		Predictions	
Hypotheses	**Water**	**Volume and/or sugar concentration**	**Rhythm**
H1 The volume and/or sugar concentration of nectar will increase or decrease as the amount of water received by the plant increases or decreases.			**No**
H2 Nectar volume and/or sugar concentration will not be affected by the amount of water received by the plant.			**Yes**

The results established in GH2 B showed a slight variation in the amplitude of the rhythms for nectar volume and sugar concentration, based on experimental day (watering amount) (see Figure 3 B). However, the daily trends in nectar volume and concentration established in the baseline experiments (GH1) were present during: day 1 (baseline conditions), day 13 (drought), and day 25 (baseline conditions) of GH2 B. The flowers exhibited low volume and high sugar concentration early in anthesis and finished with high nectar volume and

relatively low sugar concentration around midday. This discovery reveals that a decrease in water availability did not cause a significant change in the diel secretion pattern of nectar either in nectar volume or sugar concentration in *C.pepo*. These findings demonstrate that the trends in nectar volume and concentration in *C.pepo* persisted despite drought conditions, which leads to the partial rejection of hypothesis 1 and hypothesis 2 being partially supported. This discovery was unexpected as the plants in both trials of GH2 continued to produce nectar through the drought period despite cracked stems and the plants themselves appearing badly desiccated by the end of each experiment.

From a physiological perspective, flowering is resource intensive in terms of the water and carbohydrates needed for bud expansion, flower opening, and nectar production (Mohan Ram and Rao 1984; Galen et al. 1999). Furthermore, it has been demonstrated that drought stress has been linked to a reduction in the rate of photosynthesis (Dietz and Heber, 1983). Therefore, for *C.pepo* to continue to invest in reproduction despite low moisture levels, vital resources must be diverted from use in the vegetative body for growth and maintenance and transported into the developing blossoms (Reekie and Bazzaz 2005).

Clearly, continuing to divert resources away from the vegetative body such as in drought conditions will negatively affect the plants' long-term ability to survive. Therefore, it seems plausible, under the assumption that the plant will optimize fitness, that reproduction must be greatly improved in order for evolution to favor this adaptation.

From the state point of reproductive fitness, continuing to secret nectar in male flowers despite drought stress may be highly adaptive for *C.pepo*. Plants face a stochastic environment with daily and seasonal changes in the environment. However, a plant species that can invest a disproportionate amount of resources into reproduction in the middle of drought conditions

should achieve higher rates of visitation leading to an increase in the rate of pollination (Carroll et al. 2001). At this point, it is unclear how drought stress will affect nectar production in female flowers of *C.pepo*. It is reasonable to assume, however, that limited resources may hinder the plant's ability to produce viable seeds. Therefore, it is plausible that for *C.pepo* nectar production in female flowers may be affected by water availability, although empirical study is needed. On the other hand, continuing to invest resources into nectar production in male blossoms despite drought conditions may be highly advantageous; increasing the frequency of pollinator visits to male squash flowers should aid in pollen dispersion, therefore, improving reproductive fitness.

For *C.pepo* to continue to secrete highly concentrated nectar early in anthesis despite stochastic environmental conditions, which is favorable for attracting honey bees, which prefer highly concentrated nectar. Butler (1945) found that honey bees use the sugar concentration of nectar as a major deciding factor in which species of plant to exploit, preferring nectars with the highest sugar concentration available. This is in agreement with findings by Seeley et al. (1991) in which honey bee foragers preferentially visited the artificial feeder with the highest sucrose concentration. Attracting pollinators early in anthesis is vital to pollination success in *C.pepo*. Unlike species with pollen grains that dehydrate before anthesis, squash pollen is very vulnerable to desiccation during anthesis. Nepi and Pacini (1993) discovered that a 20% loss in water does irreversible damage to the pollen grain; furthermore, pollen viability starts very high at the beginning of anthesis (92%) and drops to 75% by midday.

In all of our field experiments, honey bees began to forage on squash early in anthesis, just after sunrise, and concluded after the flowers had closed for the day. These results are in agreement with previous work on *C.pepo* (Nepi and Pacini 1993). In the presence of heavy

competition, the honey bees were observed arriving early (0700 and 0800 hrs, respectively) with arrival numbers greatest in the middle of anthesis (0800 and 1000 hrs, respectively). This same foraging pattern was seen in a study conducted by Canto-Aguilar and Parra-Tabla (2000). They found that honey bees foraging on *C. moschata*, although facing competition from squash bees (*Peponapis limitaris*), essentially focused on a window 2-3 hrs after the flowers had opened, which was the time period with the highest nectar secretion rate.

In our study (FE 1), the time of peak foraging activity (0800 hrs, Figure 7) was the time period in which it appears sugar secretion rate peaked (from 0730 to 0900 hrs, Figure 6) in the veiled flowers. Encountering heavy competition, preferentially foraging during the time of peak nectar sugar production would give the foragers the highest probability of obtaining highly profitable floral rewards. Prior studies have demonstrated that honey bee workers forage using a strategy that maximizes energy gain / energy expended (Schmid-Hempel 1985). From the vantage point of optimal foraging theory (for review see Pyke 1984), eusocial organisms seek to optimize foraging behavior to yield the greatest fitness advantage to the colony. To accomplish an increase in foraging efficiency, workers adjust their foraging behavior (i.e. level of crop filling, timing of visits) in order to maximize energy gain per foraging trip (Seeley 1995).

Due to the lack of accurate nectar readings for open flowers in our field experiments (FE 1 and 2), we were not able to determine what factor (nectar volume, sugar concentration, first availability of nectar, or sugar intake rate) the bees used to trigger their time-memory. Therefore, due to this limitation, hypotheses 1, 2, 3, and 4 were not tested.

In FE 1, heavy fog affected the anticipatory behavior of the honey bee foragers, which showed an arrival pattern that was very different from FE 2. There was a complete absence of forager arrivals observed during a 2 hr period in which heavy fog enveloped the field. Honey

bee arrivals began early in anthesis but did not peak until 1100 hrs, which was after the fog had dissipated. On the other hand in FE 2, forager visits peaked during the 0745 sample time, with the number of arrivals declining at each subsequent sample time (Figure 13). This same trend in anticipatory behavior, seen in FE 2, has been seen in numerous time-memory studies done using artificial feeders (Beling 1929; Wahl 1932; Moore and Rankin 1983).

Because of our low sample size in FE 2, the Tukey post hoc analysis showed that the sample times were not significantly different from each other. At this point, it is unclear what the bees used to trigger their time-memory. It appears as though they may have been cueing on the peak of sugar concentration (supporting hypothesis 1), which according to our veiled flowers was highest early in anthesis (see Figure 5), or the first availability of nectar (supporting hypothesis 3). Unfortunately, we are not able to discriminate between these hypotheses (1 and 3) as the time period of highest concentration was also the time the nectar was first available.

As stated above, for honey bee foragers, a major component in evaluating the profitability of a food source is the sugar concentration of the nectar (Butler 1945; Seeley et al. 1991). To form her time-memory during the period of highest sugar concentration would allow each forager to visit *C.pepo* blossoms during the time of peak profitability. On the other hand, cueing on the onset of anthesis would also be highly favorable for the honey bee colony. In order to efficiently exploit a floral source, it would be advantageous for the hive to be able to deploy the maximum number of foragers at the onset of the presentation of floral rewards (Moore and Rankin 1983). This would allow the greatest number of foragers to exploit the floral source for the longest duration of time. Furthermore, the closer to the beginning of anthesis that a highly profitable source is discovered the more time employed foragers will have to advertise this lucrative site to the unemployed foragers in the hive. In addition, the foragers' ability to

time foraging excursions allows honey bees to better compete against other nectar collectors. Describing the anticipatory behavior of foraging honey bees to floral sources, Karl von Frisch stated that being early to a food source is highly adaptive in an environment full of hungry competitors (1967).

Further Research

Our preliminary results showed that honey bee foragers arrived early in anthesis, possibly cueing on either the peak in sugar concentration or the first availability of nectar in *C.pepo*. This finding will need to be further established with more trials because these results were obtained from only one successful replication. Additional work needs to be conducted to determine whether foragers cue their time-memory on the first availability of nectar or the peak sugar concentration of nectar.

The simulated rain event never actually raised the level of available water past baseline conditions. Therefore, we were not able to link the delay in honey bee time-memory extinction following a rain event to a rebound on nectar quality, nectar volume, or sugar concentration. Future studies in which the water level is significantly increased will be required to test this possibility.

The results of our current study indicate that *C. pepo* exhibits an intrinsic robust rhythm of nectar secretion that alters only slightly in response to variation in environmental variables. Further testing under constant conditions (LL or DD) would be required in order to accurately determine whether nectar secretion in *C.pepo* is truly governed by a circadian clock.

As seen in the study by Goldingay on *Corymbia gummifera* not all plant species possess a diel pattern of nectar secretion (2005). Studies investigating what other plant species possess a

diurnal secretion pattern of nectar production would also be beneficial to better understanding pollinator behavior. The results of this study have opened up more avenues of research, as more questions have yet to be answered. For example, what other plant species exhibit robust nectar rhythms that persist in spite of variation in water availability such as those discovered in *C.pepo*? Is this ability unique to *C.pepo* or has it evolved in other species as well?

As is usual in the realm of science, this study has led to more questions than answers, opening up new avenues for future research.

REFERENCES

Beling I 1929: Über das zeitgedächtnis der Bienen. Z. Vergl. Physiol. 9: 259-338.

Boose DL 1997: Sources of variation in floral nectar production rate in *Epilobium canum* (Onagraceae): Implications for natural selection. Oecologia 110: 493-500.

Brandenburg A, Dell'Olivo A, Bshary R, Kuhlemeier 2009: The sweetest thing Advances in nectar research. Current Opinion in Plant Biology 12:1-5.

Butler CG 1945: The influence of various physical and biological factors of the environment on honeybee activity. An examination of the relationship between activity and nectar concentration and abundance. Journal of Experimental Biology 21: 5-12.

Buttel-Reepen HV 1900: Sind die Bienen Reflexmaschinen? Leipzig.

Canto-Aguilar MA & Parra-Tabla V 2000: Importance of conserving alternative pollinators: assessing the pollination efficiency of the squash bee, *Peponapis limitaris* in *Cucurbita moschata*. Journal of Insect Conservation 4: 203-210.

Carroll AB, Pallardy SG, Galen S 2001: Drought stress, plant water status, and floral trait expression in fireweed, *Epilobium angustifolium* (Onagraceae). American Journal of Botany 88: 438-446.

Castellanos MC, Wilson P, Thomson JD 2002: Dynamic nectar replenishment in the flowers of *Penstemon* (Scrophulariaceae). American Journal of Botany 89:111-118.

Corbet SA, Willmer PG, Beament JWL, Unwin DM, Prys-Jones OE 1979: Post-secretory determinants of sugar concentration in nectar. *Plant Cell and Environment* (2): 293-308.

Corbet SA & Delfosse ES 1984: Honeybees and the nectar of *Echium plantagiueum* L. in southeastern Australia. Australian Journal of Ecology 9: 125-139.

Dietz KJ & Heber U 1983: Carbon dioxide gas exchange and the energy status of leaves of *Primula palinuri* under water stress. *Planta* 158: 349-356.

Forel, A. 1910: Das Sinnesleben der Insekien. Munich.

Galen C, Sherry RA, Carroll AB 1999: Are flowers physiological sinks or faucets? Costs and correlates of water use by flowers of *Polemonium viscosum*. Oecologia 118: 461-470.

Goldingay RL 2005: Is there a diel pattern of nectar secretion in the Red Bloodwood *Corymbia gummifera*? *Cunninghamia* 9: 325-329.

Kearns CA & Inouye DW 1993: Techniques for pollination biologists. University Press of Colorado, Niwot, Colorado.

Kleber E 1935: Hat das Zeitgedächtnis der Bienen biologische Bedeutung? Z. Vergl. Physiol. 22: 221-262.

Koltermann R 1971: 24-Std-Periodik in der Langzeiterrinerung an Duft- und Farbsingnale bei der Honigbiene. Z. Vergl. Physiol. 75: 49-68.

Mohan Ram HY & Rao IV 1984: Physiology of flower bud growth and opening. Proc. Indian Acd. Sci. (Plant Sci.). 93: 253-274.

Moore D, Angel JE, Cheeseman IM, Fahrbach SE, Robinson GE 1998: Timekeeping in the honey bee colony: integration of circadian rhythms and division of labor. Behav. Ecol. Sociobiol. 43:147-160.

Moore D & Rankin MA 1983: Diurnal changes in the accuracy of the honeybee foraging rhythm. Biol. Bull. 164: 471-482.

Moore-Ede M, Sulzman F, Fuller C 1984: The clocks that time us. Harvard University Press.

Nepi M & Pacini E 1993: Pollination, pollen viability and pistil receptivity in *Cucurbita pepo*. Annals of Botany 72: 52 -536.

Nepi M, Guarnieri M, Pacini E 2001: Nectar secretion, reabsorption, and sugar composition in male and female flowers of *Cucurbita pepo*. International Journal of Plant Sciences 162: 353-365.

Pacini E, Nepi M, Vesprini JL 2003: Nectar biodiversity: a short review. Plant Systematics and Evolution 238: 7-21.

Pleasants JM, 1983: Nectar production patterns in *Ipomopsis aggregata* (Polemoniaceae). American Journal of Botany 70:1468-1475.

Pyke GH, 1984: Optimal Foraging Theory: A Critical Review. Ann. Rev. Ecol. Syst. 15:523-575.

Rabinowitch HD, Fahn A, Meir T, Lensky Y 1993: Flower and nectar attributes of pepper (*Capsicum annuum* L.) plants in relation to their attractiveness to honeybees (*Apis mellifera* L.). *Annals of Applied Biology* 123: 221-232.

Reekie EG & Bazzaz FA: 2005. Reproductive allocation in plants. Academic Press

Saunders DS with Steel CGH, Vafopoulou X, Lewis RD 2002: Insect Clocks. Elsevier Science B.V., Amsterdam, the Netherlands.

Schmid-Hempel P, 1985: How do bees choose flight direction while foraging? Physiol Entomol 10:439-442.

Schutlz DJ, Huang ZY, Robinson GE 1998: Effects of colony food shortages on behavioral development in honey bees. Behav. Ecol. Sociobiol. 42: 295-303.

Seeley TD, Camazine S, Sneyd J 1991: Collective decision-making in honey bees: how colonies choose among nectar sources. Behav. Ecol. Sociobiol. 28: 277-290.

Seeley TD 1994: Honey bee foragers as sensory units of their colonies. Behav. Ecol. Sociobiol. 34: 51-62.

Seeley TD 1995: The wisdom of the hive: the social physiology of honey bee colonies. Harvard University Press, Cambridge Mass.

Southwick EE 1984: Photsynthate allocation to floral nectar: a neglected energy investment. Ecology 65: 1775-1779.

von Frisch K 1967: The dance language and orientation of bees. The Belknap Press of Harvard University Press, Cambridge Mass.

Wahl O 1932: Neue Untersuchungen über das Zeitgedächtnis der Bienen. *Z. vergl. Physiol.* 16: 529-589.

Wahl O 1933: Beitrag zur Frage des biologischen Bedeutung des Zeitgedächt-nisses der Bienen. *Z. vergl. Physiol.*18: 709 -717.

Weather Underground. (2008). Retrieved January 26, 2009, from Weather Underground: www.wunderground.com

Winston ML 1987: The Biology of the Honey Bee. Harvard University Press, Cambridge, UK.

Wyatt R, Broyles SB, Derda GS 1992: Environmental influences on nectar production in milkweeds (*Asclepias syriaca* and *A. exaltata*). *American Journal of Botany* 79: 636-642.

Zimmerman M 1983: Plant reproduction and optimal foraging: experimental nectar manipulations in *Delphinium nelsonii*. *Oikos* 41: 57-63.

APPENDICES

Appendix A
Location of Greenhouse at ETSU

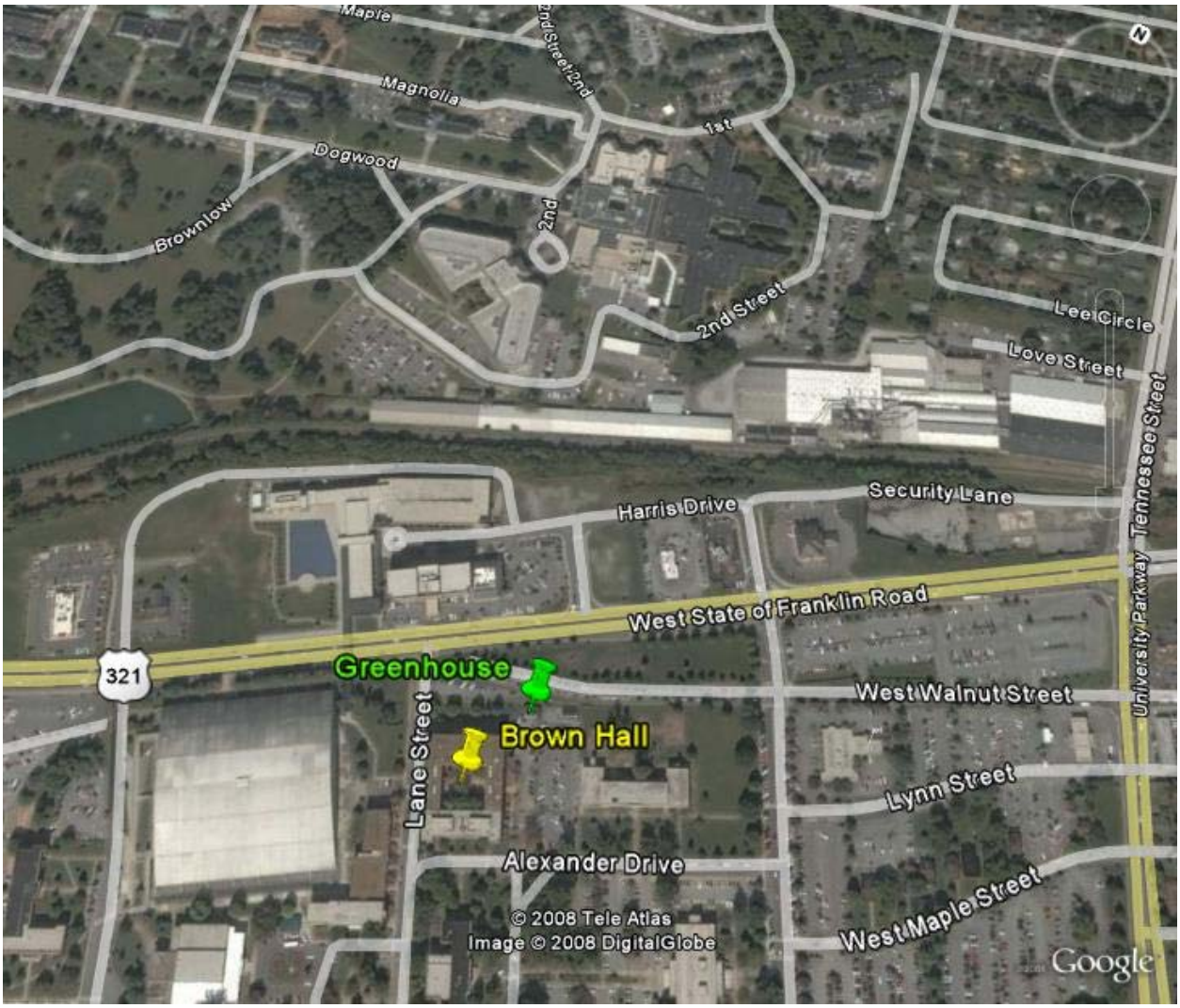


Figure 14 Location of greenhouse on the main campus of ETSU.

Appendix B
Internal Structure of the Greenhouse

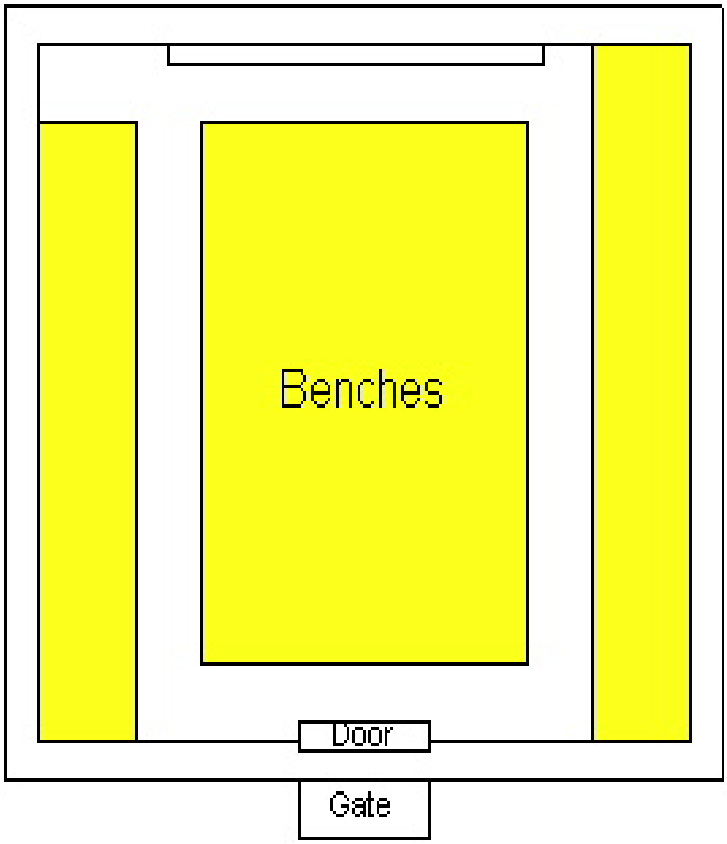


Figure 15 Bench top set-up of the greenhouse.

Appendix C
Field Set-Up for Kingsport Experiments

Figure 16 Squash field set-up in Kingsport, TN.

Appendix D
Transformation of Dependent Variable “Volume” for GH1 B.

Univariate Analysis of Variance: volume = sample time + experimental day

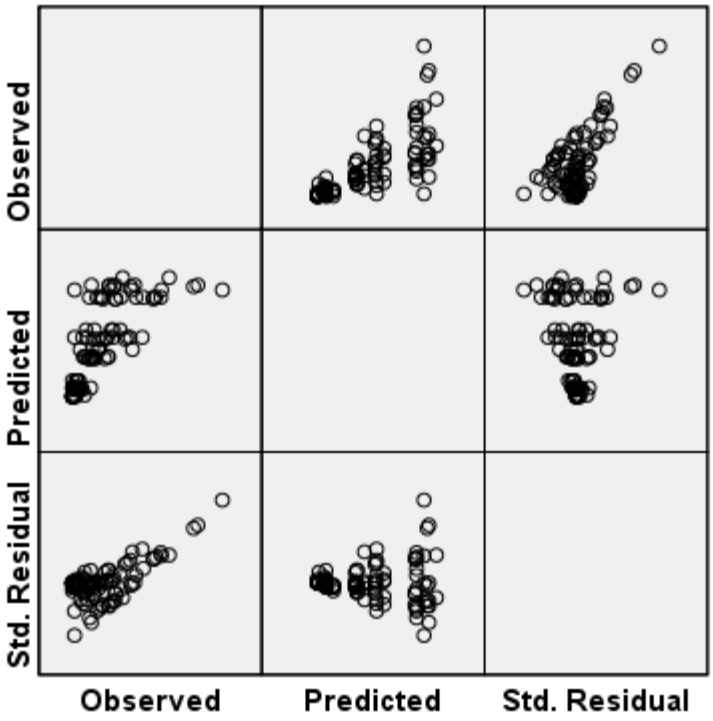
Tests of Between-Subjects Effects

Dependent Variable:Volume

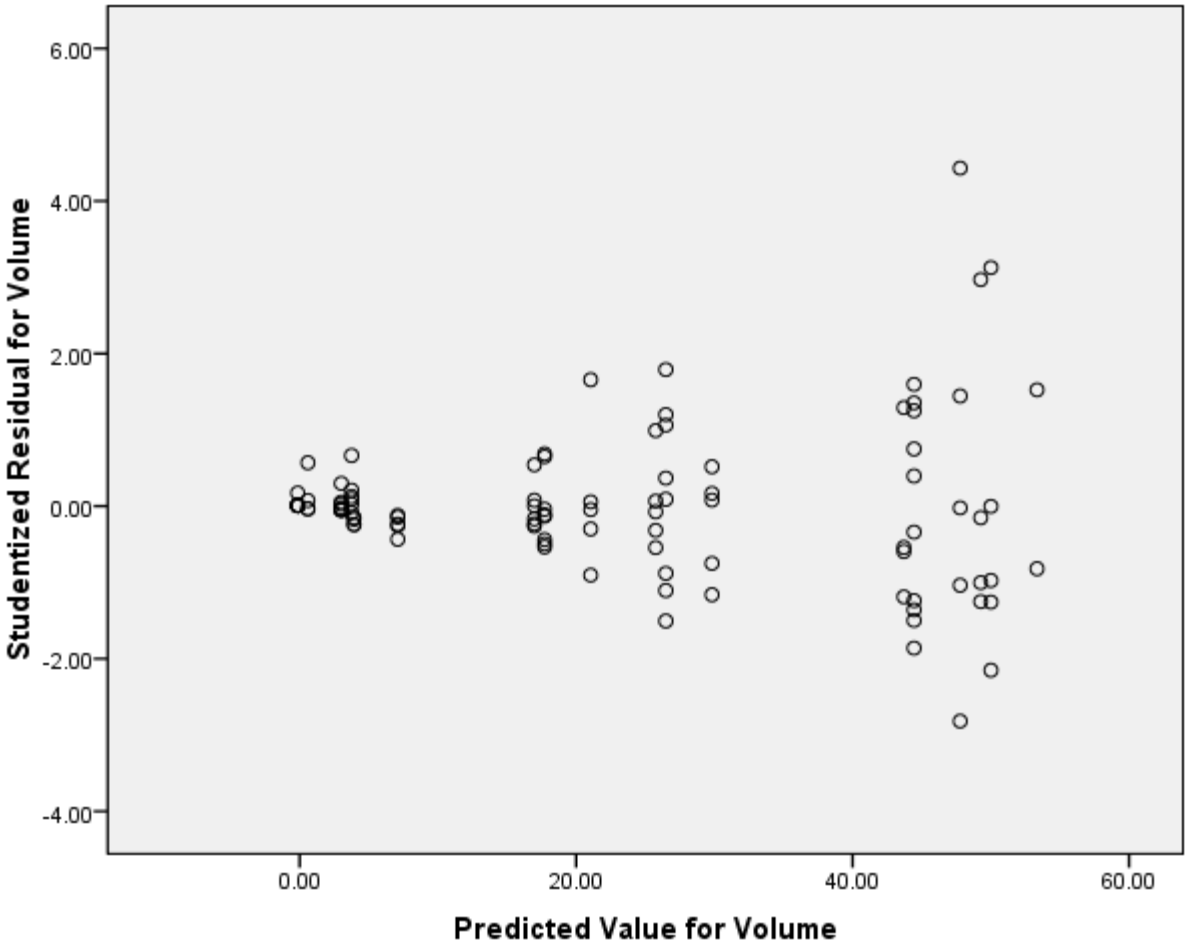
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	32883.502[a]	7	4697.643	16.151	.000
Intercept	57640.181	1	57640.181	198.174	.000
Sampletime	31442.490	5	6288.498	21.621	.000
Day	270.524	2	135.262	.465	.630
Error	27340.544	94	290.857		
Total	114339.248	102			
Corrected Total	60224.046	101			

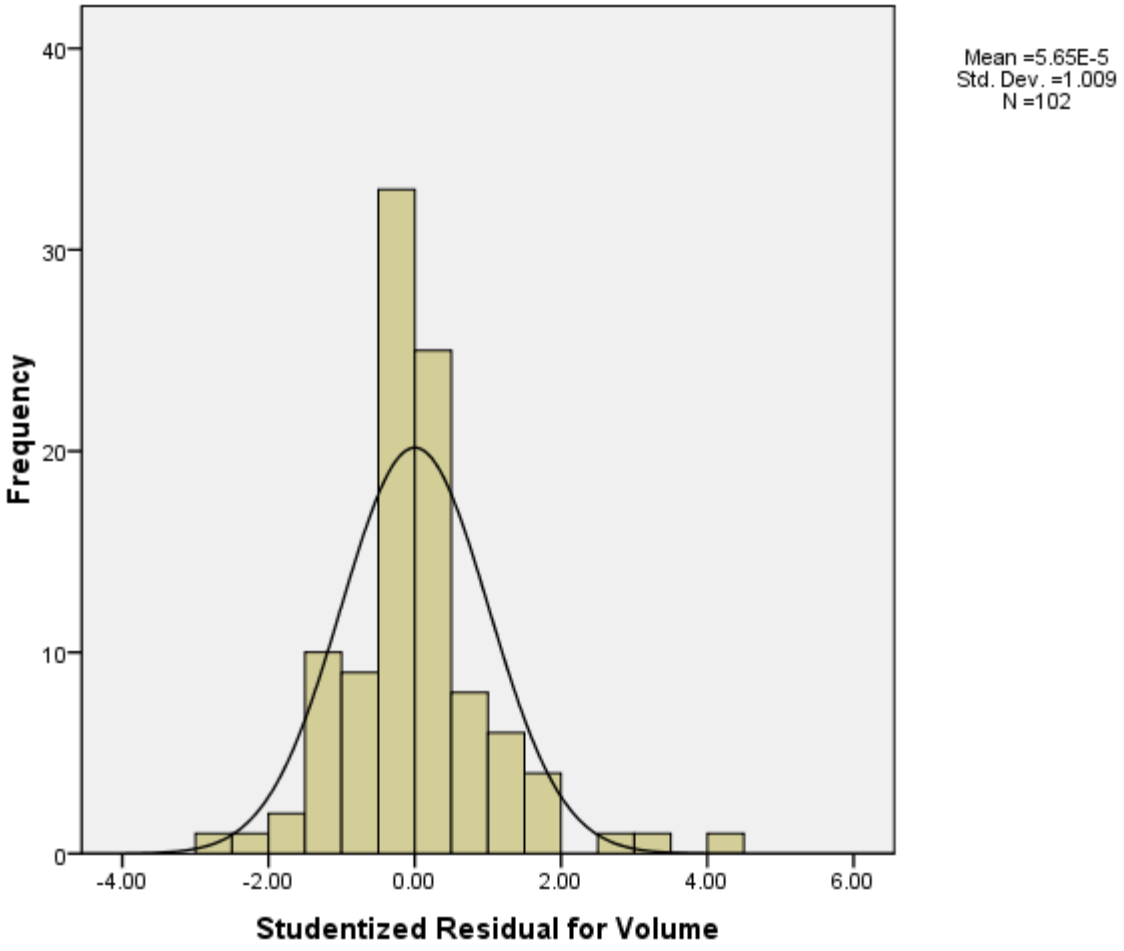
a. R Squared = .546 (Adjusted R Squared = .512)

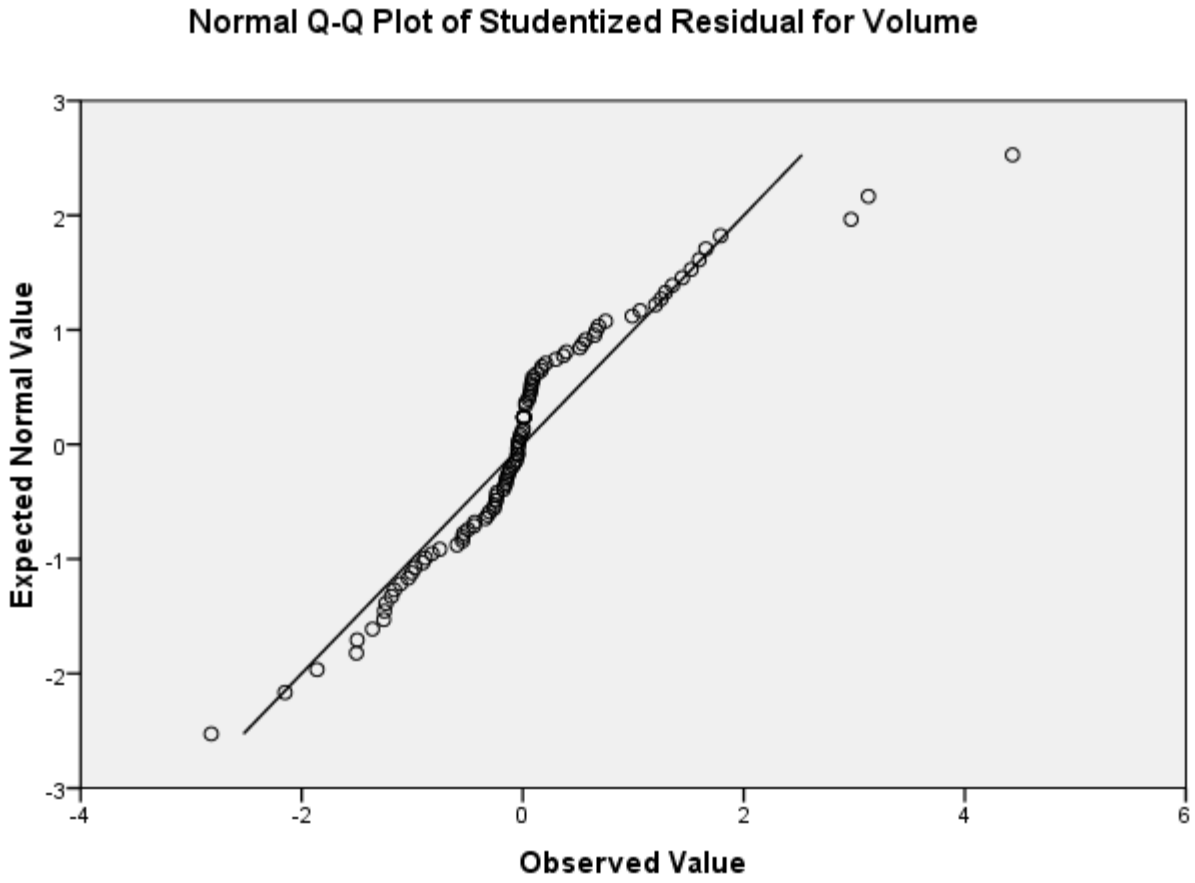
Dependent Variable: Volume

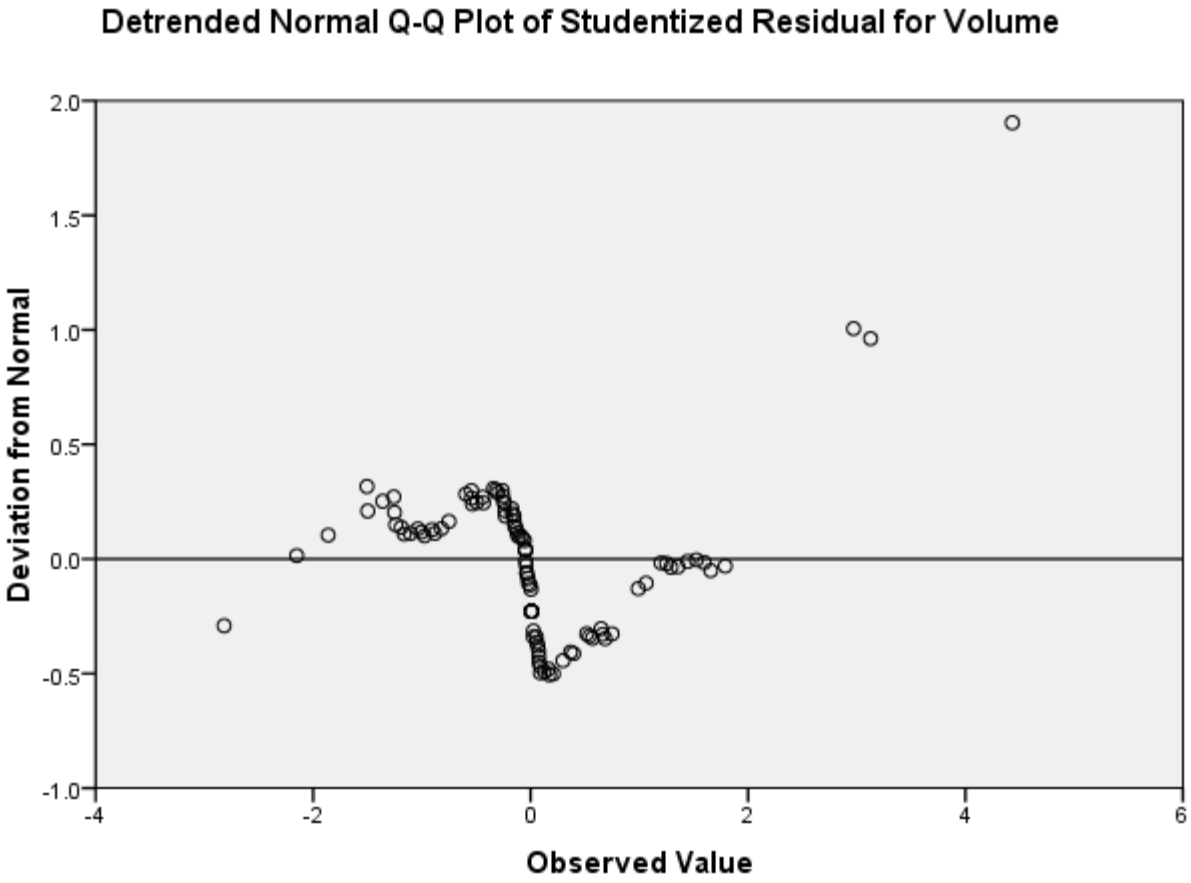


Model: Intercept + Sampletime + Day


Studentized Residual for Volume
6.00
4.00
2.00
0.00
-2.00
-4.00
0.00
20.00
40.00
60.00
Predicted Value for Volume


Mean =5.65E-5
Std. Dev. =1.009
N =102
Frequency
40
30
20
10
0
-4.00
-2.00
0.00
2.00
4.00
6.00
Studentized Residual for Volume


Normal Q-Q Plot of Studentized Residual for Volume
Expected Normal Value
Observed Value
3
2
1
0
-1
-2
-3
-4
-2
0
2
4
6


Detrended Normal Q-Q Plot of Studentized Residual for Volume
Deviation from Normal
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-4
-2
0
2
4
6
Observed Value

Univariate Analysis of Variance: Square volume = sample time + experimental day

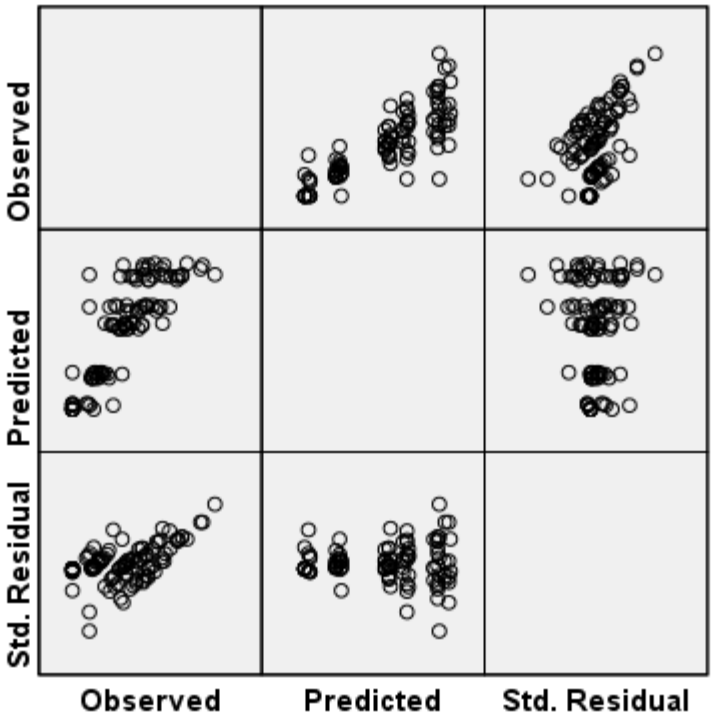
Tests of Between-Subjects Effects

Dependent Variable:Sq_volume

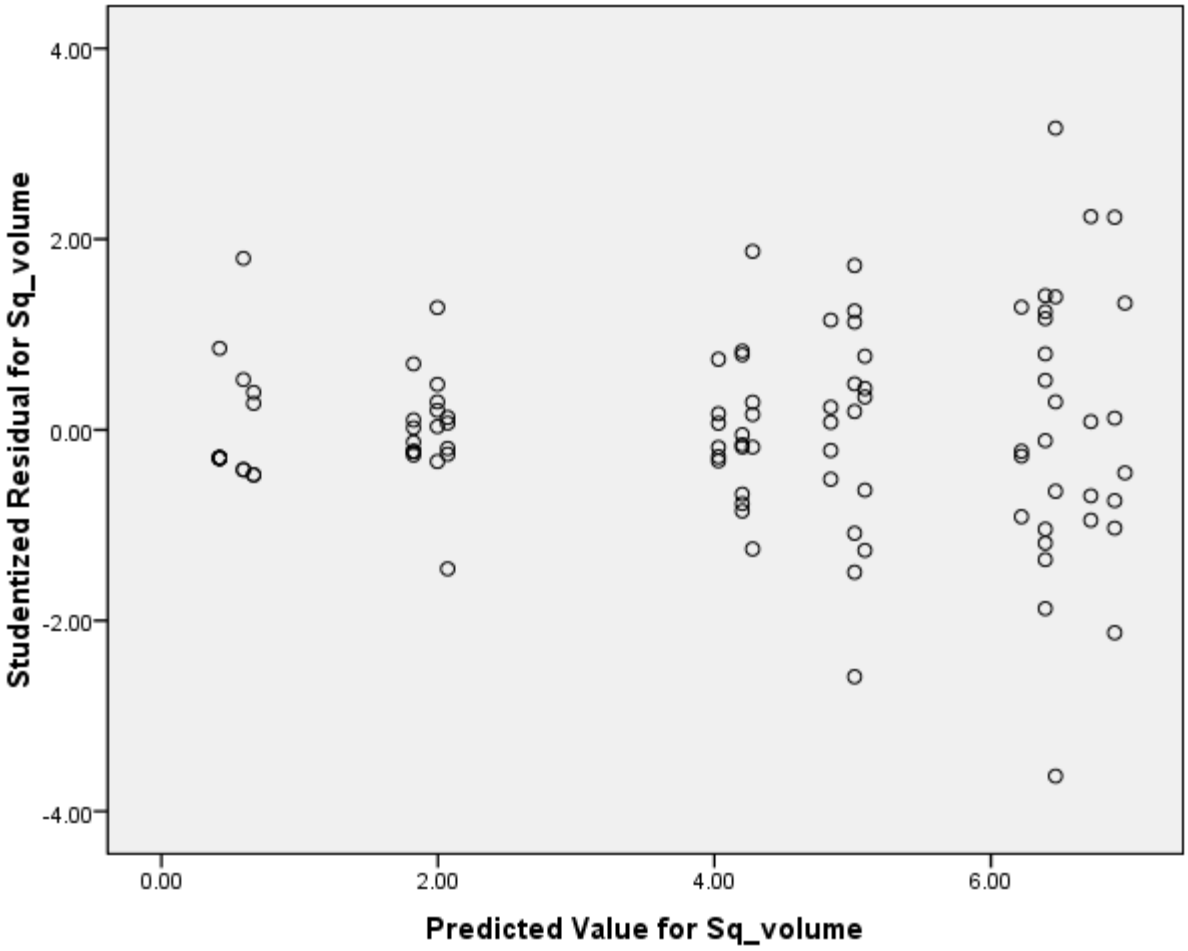
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	488.951[a]	7	69.850	31.775	.000
Intercept	1627.122	1	1627.122	740.189	.000
Sampletime	462.830	5	92.566	42.109	.000
Day	.990	2	.495	.225	.799
Error	206.636	94	2.198		
Total	2349.415	102			
Corrected Total	695.586	101			

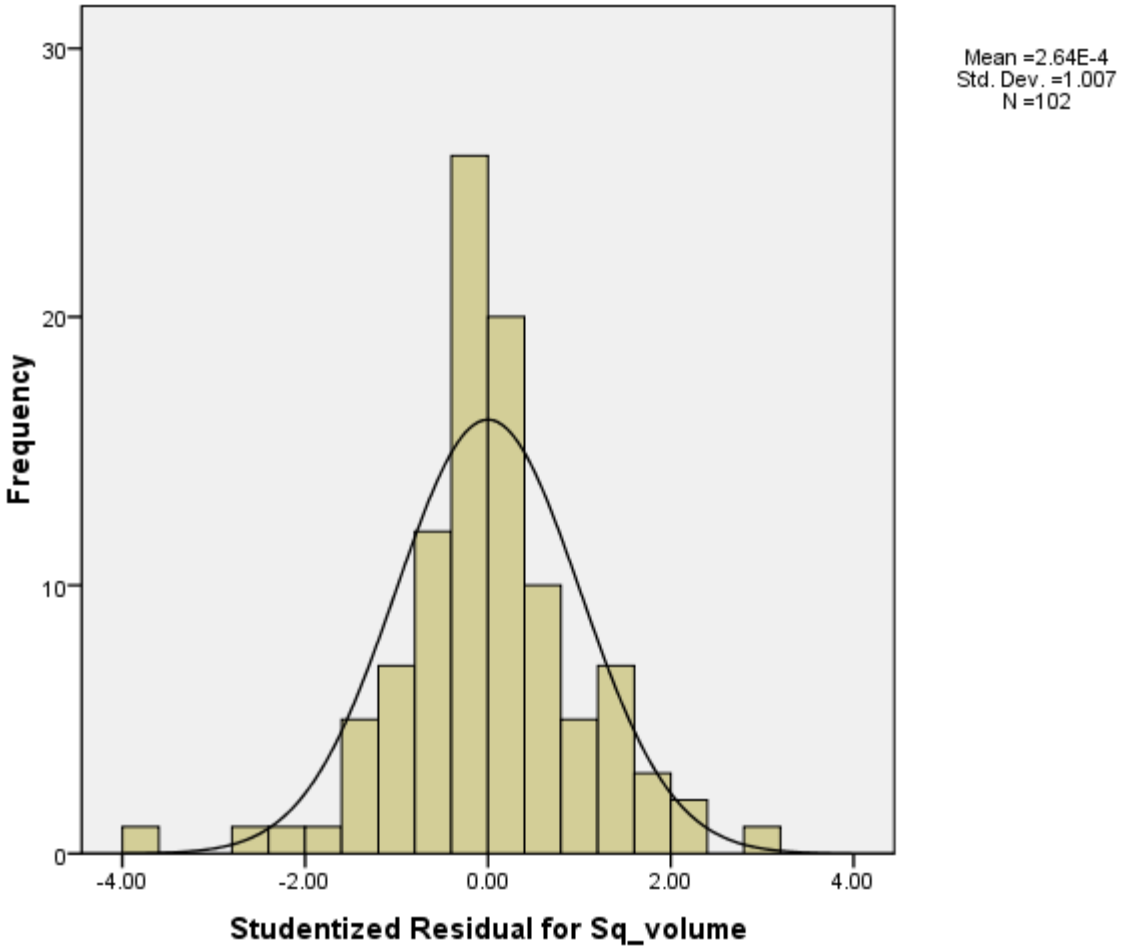
a. R Squared = .703 (Adjusted R Squared = .681)

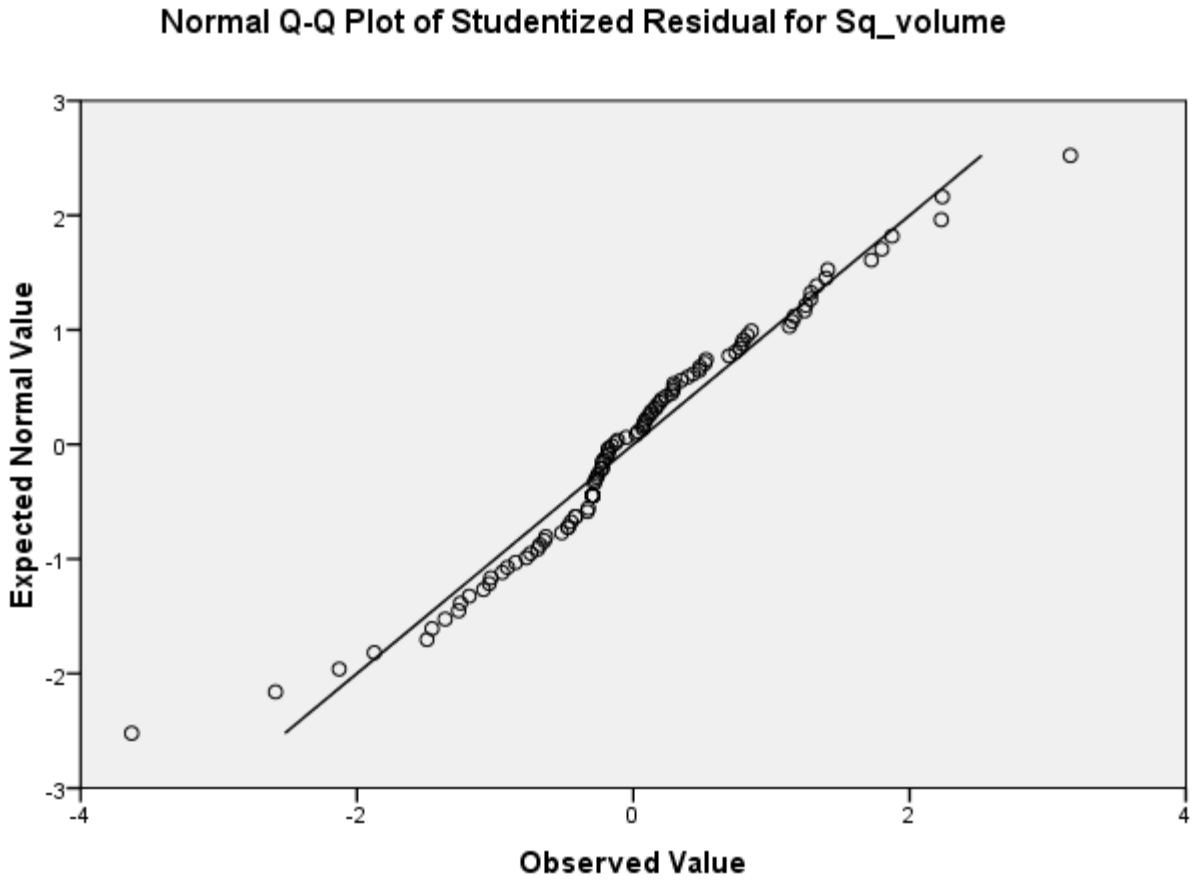
Dependent Variable: Sq_volume

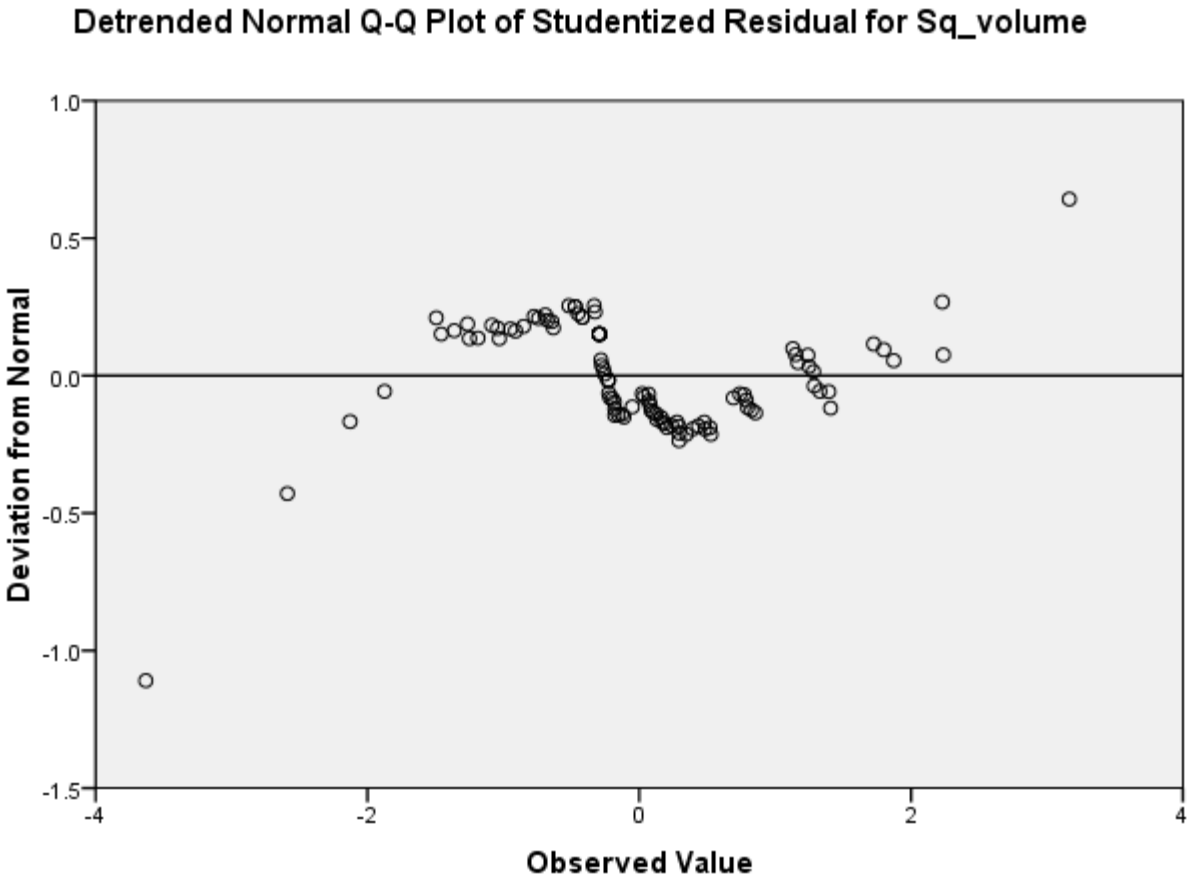


Model: Intercept + Sampletime + Day


Studentized Residual for Sq_volume
4.00
2.00
0.00
-2.00
-4.00
0.00
2.00
4.00
6.00
Predicted Value for Sq_volume


Mean =2.64E-4
Std. Dev. =1.007
N =102
Frequency
30
20
10
0
-4.00
-2.00
0.00
2.00
4.00
Studentized Residual for Sq_volume


Normal Q-Q Plot of Studentized Residual for Sq_volume
Expected Normal Value
Observed Value
3
2
1
0
-1
-2
-3
-4
-2
0
2
4


Detrended Normal Q-Q Plot of Studentized Residual for Sq_volume
Deviation from Normal
1.0
0.5
0.0
-0.5
-1.0
-1.5
-4
-2
0
2
4
Observed Value

Univariate Analysis of Variance: Log volume = sample time + experimental day

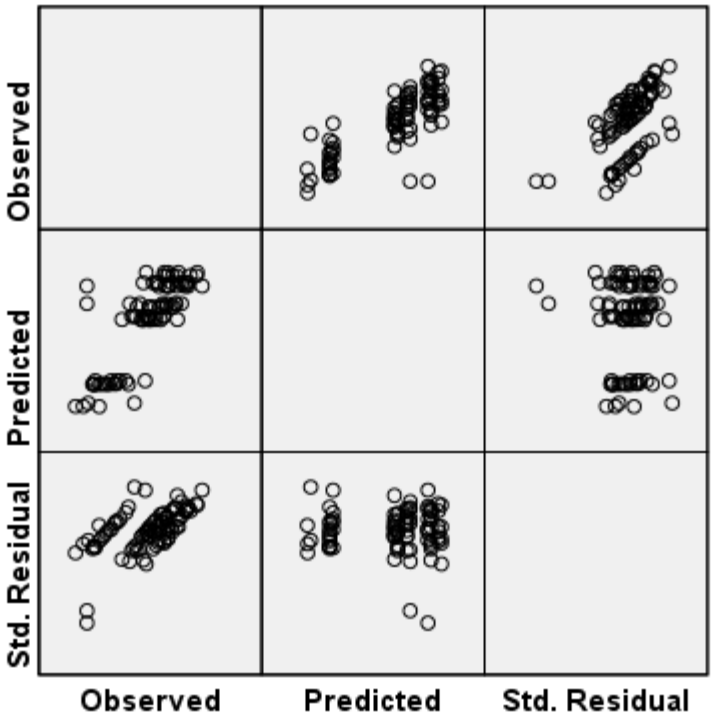
Tests of Between-Subjects Effects

Dependent Variable:Log_volume

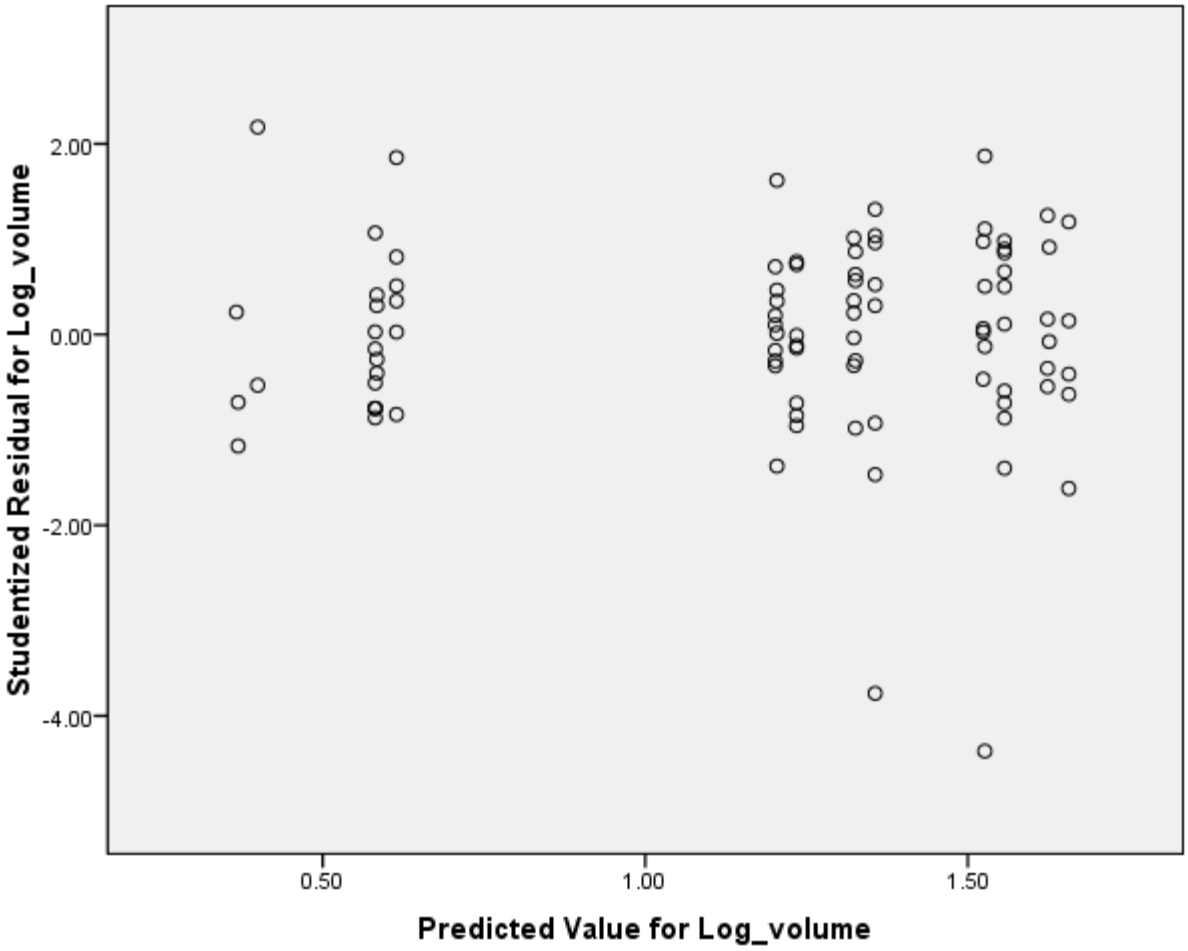
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	14.697[a]	7	2.100	21.954	.000
Intercept	85.428	1	85.428	893.270	.000
Sampletime	14.364	5	2.873	30.039	.000
Day	.022	2	.011	.115	.891
Error	7.842	82	.096		
Total	149.983	90			
Corrected Total	22.539	89			

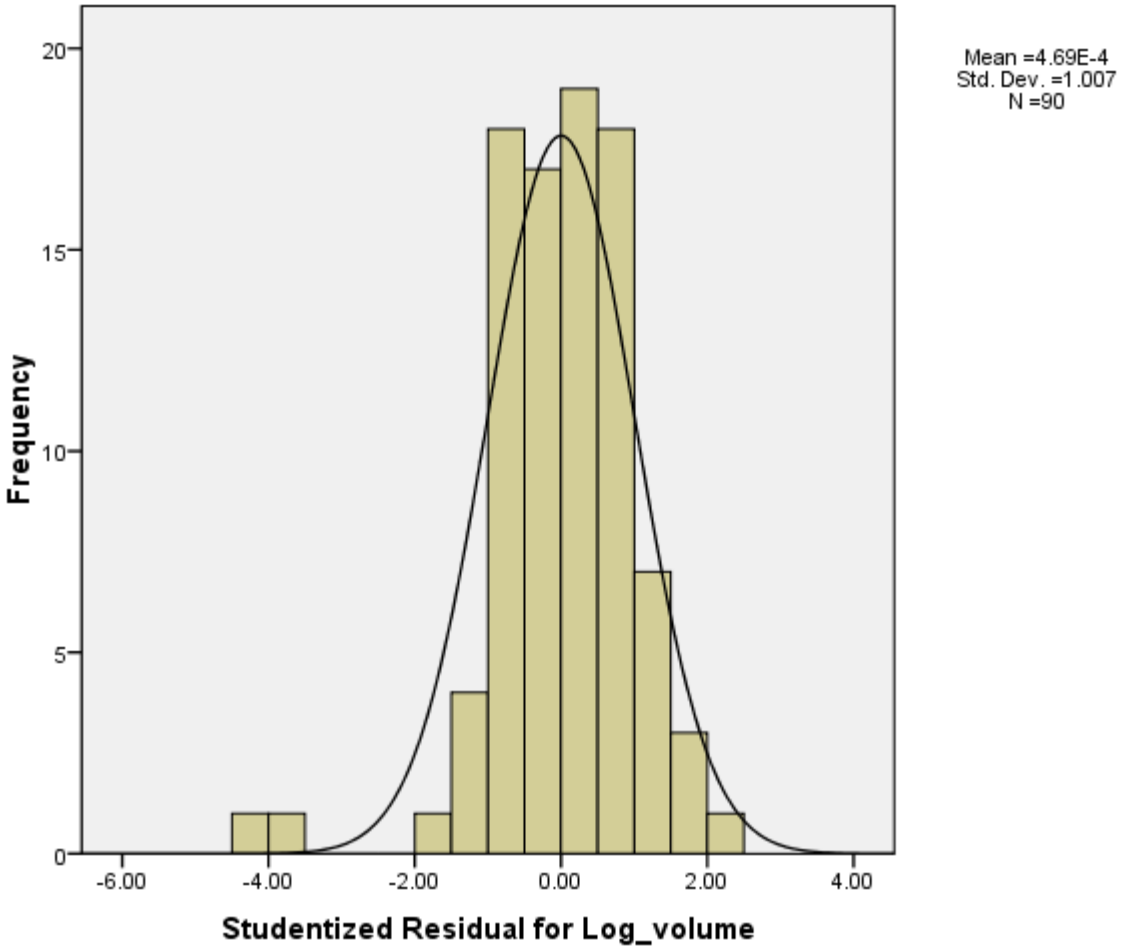
a. R Squared = .652 (Adjusted R Squared = .622)

Dependent Variable: Log_volume

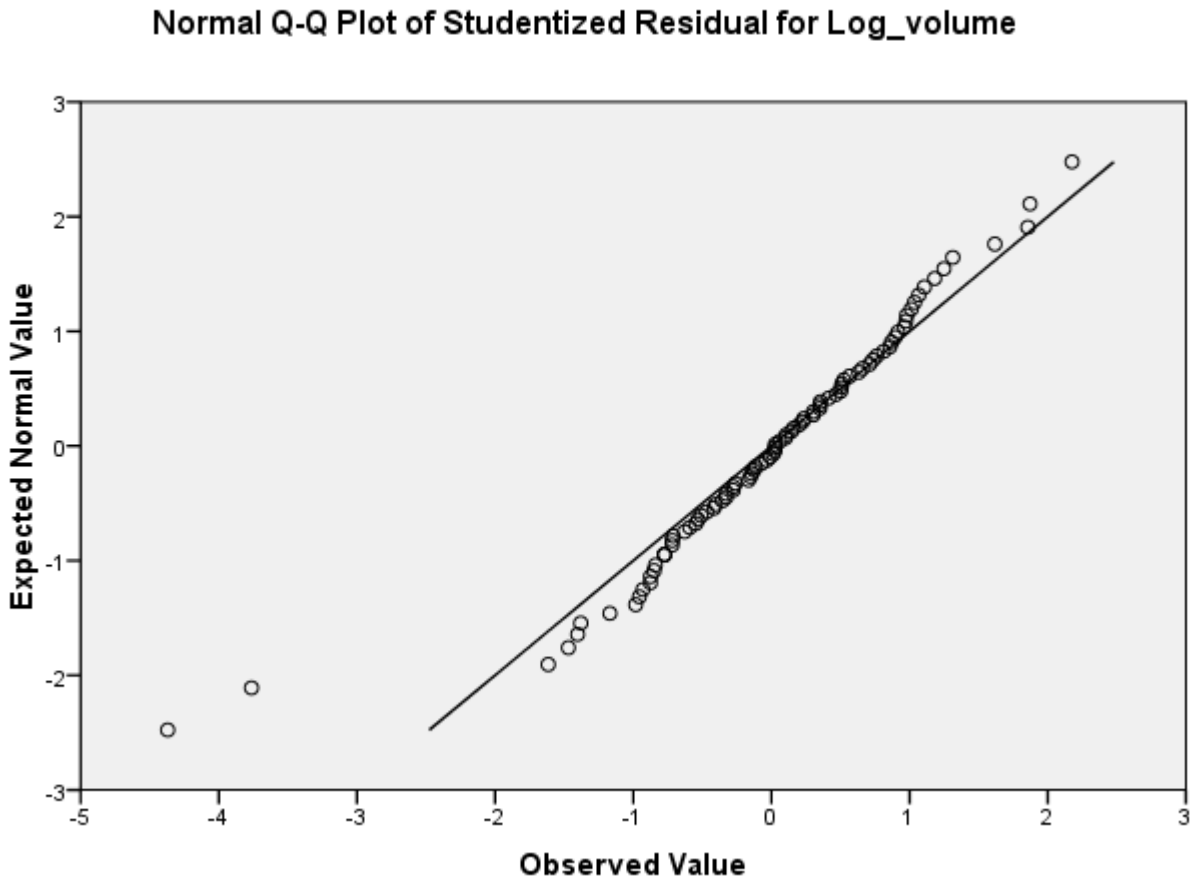


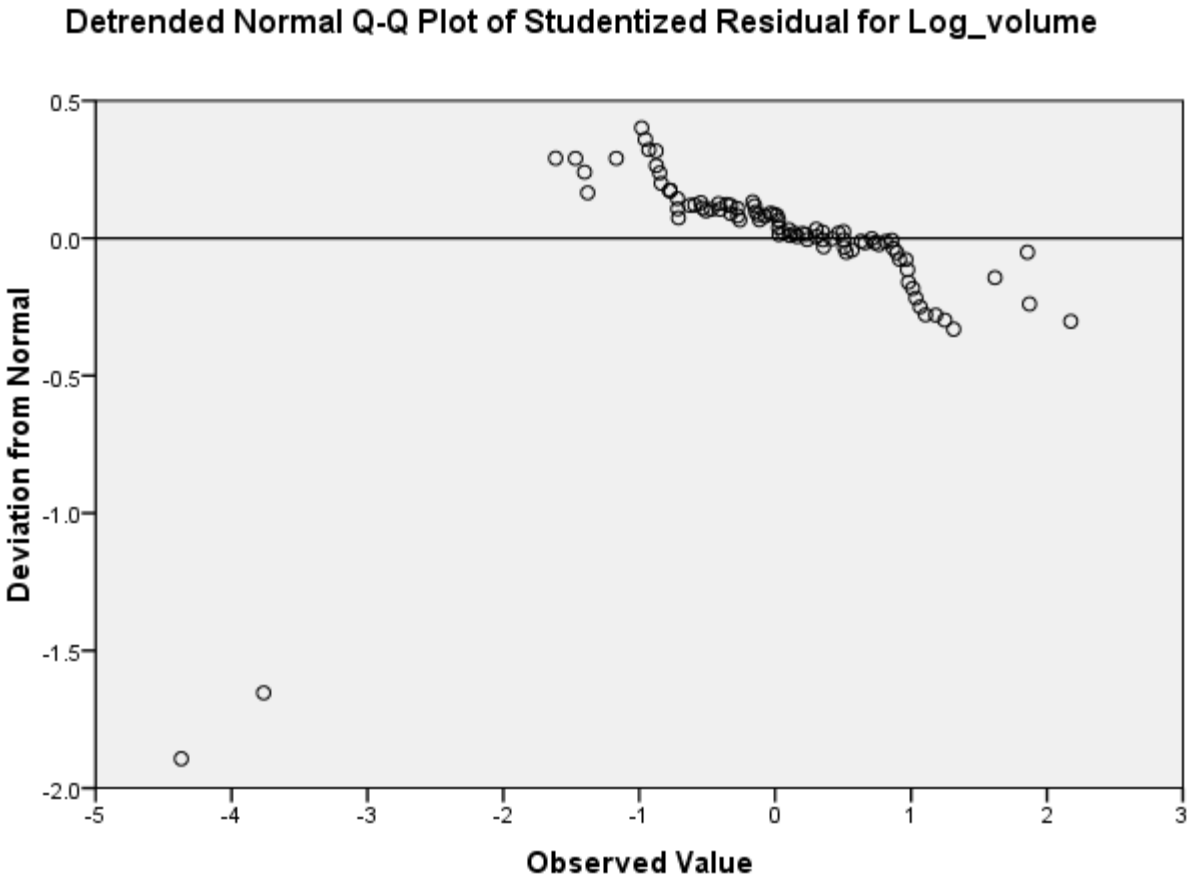
Model: Intercept + Sampletime + Day


Studentized Residual for Log_volume
2.00
0.00
-2.00
-4.00
0.50
1.00
1.50
Predicted Value for Log_volume


Mean =4.69E-4
Std. Dev. =1.007
N =90
Frequency
20
15
10
5
0
-6.00
-4.00
-2.00
0.00
2.00
4.00
Studentized Residual for Log_volume

Studentized Residual for Log_volume




Detrended Normal Q-Q Plot of Studentized Residual for Log_volume
Deviation from Normal
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-5
-4
-3
-2
-1
0
1
2
3
Observed Value

VITA

SAMUEL D. BOYD

Personal Data:	Date of Birth: September 1st, 1980 Place of Birth: Elizabethton, Tennessee
Education:	Northside Christian H.S., St. Petersburg, Florida B.S. Environmental Biology, Montreat College, Montreat, North Carolina 2005 M.S. Biology, East Tennessee State University, Johnson City, Tennessee 2009
Professional experience:	Laboratory technician, Montreat College, Montreat, North Carolina 2004 Research assistant, Montreat College, Montreat, North Carolina 2005 Biological field technician, Tennessee Valley Authority; Knoxville, Tennessee 2006
Honors and Awards:	First place poster presentation Appalachian Student research forum Alpha Chi Honor Society